

B3

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | | |
|--|--|--|--|
| (51) International Patent Classification ⁶ : C12N 15/15, A61K 38/57, C07K 14/81 | | A2 | (11) International Publication Number: WO 96/35788 |
| | | | (43) International Publication Date: 14 November 1996 (14.11.96) |
| (21) International Application Number: PCT/US96/06384 | | (US). McFADDEN, Kethleen [US/US]; Apartment E, 113 Sierra Vista Avenue, Mountain View, CA 94043 (US). GARRICK, Brett, L. [US/US]; Apartment #1, 759 Middlefield Road, Palo Alto, CA 94301 (US). | |
| (22) International Filing Date: 8 May 1996 (08.05.96) | | (74) Agents: PELTO, Don, J. et al.; Foley & Lardner, Suite 500, 3000 K Street, N.W., Washington, DC 20007-5109 (US). | |
| (30) Priority Data: 08/436,555 8 May 1995 (08.05.95) US 08/643,731 6 May 1996 (06.05.96) US | | (81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). | |
| (60) Parent Applications or Grants (63) Related by Continuation US 08/436,555 (CON) Filed on 8 May 1995 (08.05.95) US 08/643,731 (CON) Filed on 6 May 1996 (06.05.96) | | Published <i>Without international search report and to be republished upon receipt of that report.</i> | |
| (71) Applicant (for all designated States except US): SCIOS, INC. [US/US]; 2450 Bayshore Parkway, Mountain View, CA 94043 (US). | | | |
| (72) Inventors; and (75) Inventors/Applicants (for US only): WHITE, Tyler, R. [US/US]; 41600 Marigold Drive, Fremont, CA 94539 (US). DAMM, Deborah [US/US]; 711 Temescal Way, Redwood City, CA 94062 (US). LESIKAR, David, D. [US/US]; 2291 South Court, Palo Alto, CA 94301-4134 | | | |
| (54) Title: KUNITZ TYPE PROTEASE INHIBITORS | | | |
| (57) Abstract <p>Analogues of the Kunitz Protease Inhibitor (KPI) domain of amyloid precursor protein bind to and inhibit activity of serine proteases, including kallikrein, plasmin and coagulation factors such as factors VIIa, IXa, Xa, XIa and XIIa. Pharmaceutical compositions containing the KPI analogues, along with methods for using such compositions, are useful for ameliorating and treating clinical conditions associated with increased serine protease activity, such as blood loss related to cardiopulmonary bypass surgery. Nucleic acid sequences encoding these analogues and systems for expression of the peptides of the invention are provided.</p> | | | |

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|----|--------------------------|----|--|----|--------------------------|
| AM | Armenia | GB | United Kingdom | MW | Malawi |
| AT | Austria | GE | Georgia | MX | Mexico |
| AU | Australia | GN | Guinea | NE | Niger |
| BB | Barbados | GR | Greece | NL | Netherlands |
| BE | Belgium | HU | Hungary | NO | Norway |
| BF | Burkina Faso | IE | Ireland | NZ | New Zealand |
| BG | Bulgaria | IT | Italy | PL | Poland |
| BJ | Benin | JP | Japan | PT | Portugal |
| BR | Brazil | KE | Kenya | RO | Romania |
| BY | Belarus | KG | Kyrgyzstan | RU | Russian Federation |
| CA | Canada | KP | Democratic People's Republic of Korea | SD | Sudan |
| CF | Central African Republic | KR | Republic of Korea | SE | Sweden |
| CG | Congo | KZ | Kazakhstan | SG | Singapore |
| CH | Switzerland | LI | Liechtenstein | SI | Slovenia |
| CI | Côte d'Ivoire | LK | Sri Lanka | SK | Slovakia |
| CM | Cameroon | LR | Liberia | SN | Senegal |
| CN | China | LT | Lithuania | SZ | Swaziland |
| CS | Czechoslovakia | LU | Luxembourg | TD | Chad |
| CZ | Czech Republic | LV | Latvia | TG | Togo |
| DE | Germany | MC | Monaco | TJ | Tajikistan |
| DK | Denmark | MD | Republic of Moldova | TT | Trinidad and Tobago |
| EE | Estonia | MG | Madagascar | UA | Ukraine |
| ES | Spain | ML | Mali | UG | Uganda |
| FI | Finland | MN | Mongolia | US | United States of America |
| FR | France | MR | Mauritania | UZ | Uzbekistan |
| GA | Gabon | | | VN | Viet Nam |

KUNITZ TYPE PROTEASE INHIBITORS

Background of the Invention

The plasma, or serine, proteases of the blood contact system are known to be activated by interaction with negatively charged surfaces. For example, tissue injury during surgery exposes the vascular basement membrane, causing interaction of the blood with collagen, which is negatively charged at physiological pH. This induces a cascade of proteolytic events, leading to production of plasmin, a fibrinolytic protease, and consequent blood loss.

Perioperative blood loss of this type can be particularly severe during cardiopulmonary bypass (CPB) surgery, in which the patient's blood flow is diverted to an artificial heart-lung machine. CPB is an essential component of a number of life-saving surgical procedures. For example, in the United States, it is estimated that 300,000 patients every year undergo coronary artery bypass grafts involving the use of CPB.

Although necessary and generally safe, CPB is associated with a significant rate of morbidity, some of which may be attributed to a "whole body inflammatory response" caused by activation of plasma protease systems and blood cells through interactions with the artificial surfaces of the heart-lung machine (Butler et al., *Ann. Thorac. Surg.* 55:552 (1993); Edmunds et al., *J. Card. Surg.* 8:404 (1993)). For example, during extracorporeal circulation, exposure of blood to negatively charged surfaces of the artificial bypass circuit, e.g., plastic surfaces in the heart-lung machine, results in direct activation of plasma factor XII.

Factor XII is a single-chain 80 kDa protein that circulates in plasma as an inactive zymogen. Contact with negatively charged nonendothelial surfaces, like those of the bypass circuit, causes surface-bound factor XII to be autoactivated to the active serine protease factor XIIa. See Colman, *Agents Actions Suppl.* 42:125

(1993). Surface-activated factor XIIa then processes prekallikrein (PK) to active kallikrein, which in turn cleaves more XIIa from XII in a reciprocal activation reaction that results in a rapid amplification of the contact pathway. Factor XIIa can also activate the first component of complement C1, leading to production of the anaphylatoxin C5a through the classical complement pathway.

The CPB-induced inflammatory response includes changes in capillary permeability and interstitial fluid accumulation. Cleavage of high molecular weight kininogen (HK) by activated kallikrein generates the potent vasodilator bradykinin, which is thought to be responsible for increasing vascular permeability, resulting in edema, especially in the lung. The lung is particularly susceptible to damage associated with CPB, with some patients exhibiting what has been called "pump lung syndrome" following bypass, a condition indistinguishable from adult respiratory distress. See Johnson et al., *J. Thorac. Cardiovasc. Surg.* 107:1193 (1994).

Post-CPB pulmonary injury includes tissue damage thought to be mediated by neutrophil sequestration and activation in the microvasculature of the lung. (Butler et al., *supra*; Johnson, et al., *supra*). Activated factor XII can itself stimulate neutrophil aggregation. Factor XIIa-generated kallikrein, and complement protein C5a generated by Factor XIIa activation of the complement cascade, both induce neutrophil chemotaxis, aggregation and degranulation. See Edmunds et al., *supra* (1993). Activated neutrophils may damage tissue through release of oxygen-derived free-radicals, proteolytic enzymes such as elastase, and metabolites of arachidonic acid. Release of neutrophil products in the lung can cause changes in vascular tone, endothelial injury and loss of vascular integrity.

Intrinsic inhibition of the contact system occurs through inhibition of activated XIIa by C1-inhibitor (C1-INH). See Colman, *supra*. During CPB, this natural

inhibitory mechanism is overwhelmed by massive activation of plasma proteases and consumption of inhibitors. A potential therapeutic strategy for reducing post-bypass pulmonary injury mediated by neutrophil activation would, therefore, be to block the formation and activity of the neutrophil agonists kallikrein, factor XIIa, and C5a by inhibition of proteolytic activation of the contact system.

Protease inhibitor therapy which partially attenuates the contact system is currently employed clinically in CPB. Aprotinin, also known as basic pancreatic protease inhibitor (BPPi), is a small, basic, 58 amino acid polypeptide isolated from bovine lung. It is a broad spectrum serine protease inhibitor of the Kunitz type, and was first used during bypass in an attempt to reduce the inflammatory response to CPB. See Butler et al., *supra*. Aprotinin treatment results in a significant reduction in blood loss following bypass, but does not appear to significantly reduce neutrophil activation. Additionally, since aprotinin is of bovine origin, there is concern that repeated administration to patients could lead to the development of an immune response to aprotinin in the patients, precluding its further use.

The proteases inhibited by aprotinin during CPB appear to include plasma kallikrein and plasmin. (See, e.g., Scott, et al., *Blood* 69:1431 (1987)). Aprotinin is an inhibitor of plasmin (K_i of 0.23nM), and the observed reduction in blood loss may be due to inhibition of fibrinolysis through the blocking of plasmin action. Although aprotinin inhibits plasma kallikrein, (K_i of 20nM), it does not inhibit activated factor XII, and consequently only partially blocks the contact system during CPB.

Another attractive protease target for use of protease inhibitors, such as those of the present invention, is factor XIIa, situated at the very first step of contact activation. By inhibiting the proteolytic activity of factor XIIa, kallikrein production would be prevented, blocking amplification of

the contact system, neutrophil activation and bradykinin release. Inhibition of XIIa would also prevent complement activation and production of C5a. More complete inhibition of the contact system during CPB could, therefore, be achieved through the use of a better XIIa inhibitor.

Protein inhibitors of factor XIIa are known. For example, active site mutants of α_1 -antitrypsin that inhibit factor XIIa have been shown to inhibit contact activation in human plasma. See Patston et al., *J. Biol. Chem.* 265:10786 (1990). The large size and complexity (greater than 400 amino acid residues) of these proteins present a significant challenge for recombinant protein production, since large doses will almost certainly be required during CPB. For example, although it is a potent inhibitor of both kallikrein and plasmin, nearly 1 gram of aprotinin must be infused into a patient to inhibit the massive activation of the kallikrein-kinin and fibrinolytic systems during CPB.

The use of smaller, more potent XIIa inhibitors such as the corn and pumpkin trypsin inhibitors (Wen, et al., *Protein Exp. & Purif.* 4:215 (1993); Pedersen, et al., *J. Mol. Biol.* 236:385 (1994)) could be more cost-effective than the large α_1 -antitrypsins, but the infusion of high doses of these non-mammalian inhibitors could result in immunologic reactions in patients undergoing repeat bypass operations. The ideal protein XIIa inhibitor is, therefore, preferably, small, potent, and of human sequence origin.

One candidate for an inhibitor of human origin is found in circulating isoforms of the human amyloid β -protein precursor (APPI), also known as protease nexin-2. APPI contains a Kunitz serine protease inhibitor domain known as KPI (Kunitz Protease Inhibitor). See Ponte et al., *Nature*, 331:525 (1988); Tanzi et al., *Nature* 331:528 (1988); Johnstone et al., *Biochem. Biophys. Res. Commun.* 163:1248 (1989); Oltersdorf et al., *Nature* 341:144 (1989). Human KPI shares about 45% amino acid sequence identity with aprotinin. The isolated KPI domain has

been prepared by recombinant expression in a variety of systems, and has been shown to be an active serine protease inhibitor. See, for example, Sinha, et al., *J. Biol. Chem.* 265:8983 (1990). The measured *in vitro* K_i of KPI against plasma kallikrein is 45nM, compared to 20nM for aprotinin.

Aprotinin, KPI, and other Kunitz-type serine protease inhibitors have been engineered by site-directed mutagenesis to improve inhibitory activity or specificity. Thus, substitution of Lys¹⁵ of aprotinin with arginine resulted in an inhibitor with a K_i of 0.32nM toward plasma kallikrein, a 100-fold improvement over natural aprotinin. See PCT application No. 89/10374. See also Norris et al., *Biol. Chem. Hoppe Seyler* 371:3742 (1990). Alternatively, substitution of position 15 of aprotinin with valine or substitution of position 13 of KPI with valine resulted in elastase inhibitors with K_i s in the 100 pM range, although neither native aprotinin nor native KPI significantly inhibits elastase. See Wenzel et al., in: *Chemistry of Peptides and Proteins*, Vol. 3, (Walter de Gruyter, Berlin, New York, 1986); Sinha et al., *supra*. Methods for substituting residues 13, 15, 37, and 50 of KPI are shown in general terms in European Patent Application No. 0 393 431, but no specific sequences are disclosed, and no protease inhibition data are given.

Phage display methods have been recently used for preparing and screening derivatives of Kunitz-type protease inhibitors. See PCT Application No. 92/15605, which describes specific sequences for 34 derivatives of aprotinin, some of which were reportedly active as elastase and cathepsin inhibitors. The amino acid substitutions in the derivatives were distributed throughout almost all positions of the aprotinin molecule.

Phage display methods have also been used to generate KPI variants that inhibit factor VIIa and kallikrein. See Dennis et al., *J. Biol. Chem.* 269:22129 and 269:22137 (1994). The residues that could be varied in the phage

display selection process were limited to positions 9-11, 13-17, 32, 36 and 37, and several of those residues were also held constant for each selection experiment. One of those variants was said to have a K_i of 1.2nM for kallikrein, and had substitutions at positions 9 (Thr→Pro), 13 (Arg→Lys), 15 (Met→Leu), and 37 (Gly→Tyr). None of the inhibitors was tested for the ability to inhibit factor XIIa.

It is apparent, therefore, that new protease inhibitors that can bind to and inhibit the activity of serine proteases are greatly to be desired. In particular it is highly desirable to prepare peptides, based on human peptide sequences, that can inhibit selected serine proteases such as kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator. It is also highly desirable to prepare novel protease inhibitors that can ameliorate one or more of the undesirable clinical manifestations associated with enhanced serine protease activity, for example by reducing pulmonary damage or blood loss during CPB.

Summary of the Invention

The present invention relates to peptides that can bind to and preferably exhibit inhibition of the activity of serine proteases. Those peptides can also provide a means of ameliorating, treating or preventing clinical conditions associated with increased activity of serine proteases. Particularly, the novel peptides of the present invention preferably exhibit a more potent and specific (i.e., greater) inhibitory effect toward serine proteases of interest in comparison to known serine protease inhibitors. Examples of such proteases include: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly

those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

5 In achieving the inhibition of serine protease activity, the invention provides protease inhibitors that can ameliorate one or more of the undesirable clinical manifestations associated with enhanced serine protease activity, for example, by reducing pulmonary damage or
10 blood loss during CPB.

The present invention relates to protease inhibitors comprising the following amino acid sequences:

X¹-Val-Cys-Ser-Glu-Gln-Ala-Glu-X²-Gly-X³-Cys-Arg-Ala-X⁴-X⁵-X⁶-X⁷-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-
15 Lys-Cys-Ala-Pro-Phe-X⁸-Tyr-Gly-Gly-Cys-X⁹-X¹⁰-X¹¹-X¹²-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein: X¹ is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu; X² is selected from Thr, Val, Ile and Ser; X³ is
20 selected from Pro and Ala; X⁴ is selected from Arg, Ala, Leu, Gly, or Met; X⁵ is selected from Ile, His, Leu, Lys, Ala, or Phe; X⁶ is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu; X⁷ is selected from Arg, His, or Ala; X⁸ is selected from Phe, Val, Leu, or Gly;
25 X⁹ is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr; X¹⁰ is selected from Ala, Arg, or Gly; X¹¹ is selected from Lys, Ala, or Asn; and X¹² is selected from Ser, Ala, or Arg.

The invention relates more specifically to protease
30 inhibitors comprising the following amino acid sequences:

X¹-Val-Cys-Ser-Glu-Gln-Ala-Glu-X²-Gly-X³-Cys-Arg-Ala-X⁴-X⁵-X⁶-X⁷-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-
Lys-Cys-Ala-Pro-Phe-X⁸-Tyr-Gly-Gly-Cys-X⁹-X¹⁰-X¹¹-
35 X¹²-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein X¹ is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu; X² is selected from Thr, Val, Ile and Ser; X³ is selected from Pro and Ala; X⁴ is selected from Arg, Ala, Leu, Gly, or Met; X⁵ is selected from Ile, His, Leu, Lys,

Ala, or Phe; X⁶ is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu; X⁷ is selected from Arg, His, or Ala; X⁸ is selected from Phe, Val, Leu, or Gly; X⁹ is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr; X¹⁰ is selected from Ala, Arg, or Gly; X¹¹ is selected from Lys, Ala, or Asn; X¹² is selected from Ser, Ala, or Arg; provided that when X⁴ is Arg, X⁶ is Ile; when X⁹ is Arg, X⁴ is Ala or Leu; when X⁹ is Tyr, X⁴ is Ala or X⁵ is His; and either X⁵ is not Ile; or X⁶ is not Ser; or X⁹ is not Leu, Phe, Met, Tyr, or Asn; or X¹⁰ is not Gly; or X¹¹ is not Asn; or X¹² is not Arg.

Another aspect of this invention provides protease inhibitors wherein at least two amino acid residues selected from the group consisting of X⁴, X⁵, X⁶, and X⁷ defined above differ from the residues found in the naturally occurring sequence of KPI. Another aspect of this invention provides protease inhibitors wherein X¹ is Asp or Glu, X² is Thr, X³ is Pro, and X¹² is Ser. Yet another aspect of this invention provides protease inhibitors wherein X¹ is Glu, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, and X¹¹ is Asn. Another aspect of this invention provides protease inhibitors wherein X¹ is Asp, X² is Thr, X³ is Pro, X⁴ is Arg, X⁵ is Ile, X⁶ is Ile, X⁷ is Arg, X⁸ is Val, X⁹ is Arg, X¹⁰ is Ala, and X¹¹ is Lys. Another aspect of this invention provides protease inhibitors wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Asn, and X¹² is Ala. Another aspect of this invention provides protease inhibitors wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Ala, and X¹² is Arg. Another aspect of this invention provides protease inhibitors wherein X¹ is Glu, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Ala, X¹¹ is Asn, and X¹² is Arg. Another aspect of this invention provides protease inhibitors wherein X¹ is

Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Arg, X¹¹ is Asn, and X¹² is Arg. Another aspect of this invention provides protease inhibitors wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Val, Leu, or Gly, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Asn, and X¹² is Arg. Another aspect of this invention provides protease inhibitors wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Ala, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Asn, and X¹² is Arg. Another aspect of this invention provides protease inhibitors wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, Val, or Ser, X³ is Pro, X⁴ is Ala or Leu, X⁵ is Ile, X⁶ is Tyr, X⁷ His, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Ala, and X¹² is Arg.

Yet another aspect of this invention provides protease inhibitors wherein X² is Thr, and X⁴ is Ala. Another aspect of this invention provides protease inhibitors wherein X² is Thr, and X⁴ is Leu. Another aspect of this invention provides protease inhibitors wherein X² is Val, and X⁴ is Ala. Another aspect of this invention provides protease inhibitors wherein X² is Ser, and X⁴ is Ala. Another aspect of this invention provides protease inhibitors wherein X² is Val, and X⁴ is Leu. Another aspect of this invention provides protease inhibitors wherein X² is Ser, and X⁴ is Leu.

Yet another aspect of this invention provides protease inhibitors wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Leu, X⁵ is Phe, X⁶ is Lys, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Ala, and X¹² is Arg. Another aspect of this invention provides protease inhibitors wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Leu, X⁵ is Phe, X⁶ is Lys, X⁷ is Arg, X⁸ is Phe, X⁹ is Tyr, X¹⁰ is Gly, X¹¹ is Ala, and X¹² is Arg. Another aspect of this invention provides protease inhibitors wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Leu, X⁵ is Phe, X⁶ is Lys, X⁷ is Arg, X⁸ is Phe, X⁹ is Leu, X¹⁰ is Gly, X¹¹ is Ala, and X¹² is Arg.

The present invention also relates to protease inhibitors comprising the following amino acid sequences:

5 X¹-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-
Pro-Cys-X²-Ala-X³-X⁴-X⁵-X⁶-Trp-Tyr-Phe-Asp-
Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-
Tyr-Gly-Gly-Cys-Gly-Gly-Asn-Arg-Asn-Asn-
Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-
Cys-Gly-Ser-Ala-Ile,

10 wherein: X¹ is selected from Glu-Val-Val-Arg-Glu- and
Asp-Val-Val-Arg-Glu-; X² is selected from Arg and Lys; X³
is selected from Met, Arg, Ala, Leu, Ser, Val; X⁴ is
selected from Ile and Ala; X⁵ is selected from Ser, Ile,
Ala, Pro, Phe, Tyr, and Trp; and X⁶ is selected from Arg,
Ala, His, Gln, and Thr; provided that: when X² is Arg, X³
15 is Leu, and X⁴ is Ile, X⁵ cannot be Ser; and also provided
that either X³ is not Met; or X⁴ is not Ile; or X⁵ is not
Ser; or X⁶ is not Arg. Another aspect of this invention
provides protease inhibitors wherein X³ is Arg or Met,
and X⁵ is Ser or Ile. Yet another aspect of this
20 invention provides protease inhibitors wherein X⁵ is
selected from Phe, Tyr and Trp. Another aspect of this
invention provides protease inhibitors wherein X³ is Ala
or Leu.

25 A further aspect of this invention provides an
isolated DNA molecule comprising a DNA sequence encoding
a protease inhibitor of the invention. Another aspect of
this invention provides an isolated DNA molecule
comprising a DNA sequence encoding the protease inhibitor
that further comprises an isolated DNA molecule operably
30 linked to a regulatory sequence that controls expression
of the coding sequence of the protease inhibitor in a
host cell. Another aspect of this invention provides an
isolated DNA molecule comprising a DNA sequence encoding
the protease inhibitor operably linked to a regulatory
35 sequence that controls expression of the coding sequence
of the protease inhibitor in a host cell that further
comprises a DNA sequence encoding a secretory signal
peptide. That secretory signal peptide may preferably
comprise the signal sequence of yeast alpha-mating

factor. Another aspect of this invention provides a host cell transformed with any of the DNA molecules defined above. Such a host cell may preferably comprise *E. coli* or a yeast cell. When such a host cell is a yeast cell, the yeast cell may be selected from *Saccharomyces cerevisiae* and *Pichia pastoris*.

Another aspect of this invention provides a method for producing a protease inhibitor of the present invention, comprising the steps of culturing a host cell as defined above and isolating and purifying said protease inhibitor.

A further aspect of this invention provides a pharmaceutical composition, comprising a protease inhibitor of the present invention together with a pharmaceutically acceptable sterile vehicle.

An additional aspect of this invention provides a method of treatment of a clinical condition associated with increased activity of one or more serine proteases, comprising administering to a patient suffering from said clinical condition an effective amount of a pharmaceutical composition comprising a protease inhibitor of the present invention together with a pharmaceutically acceptable sterile vehicle. That method of treatment may preferably be used to treat the clinical condition of blood loss during surgery.

Yet another aspect of this invention provides a method for inhibiting the activity of serine proteases of interest in a mammal comprising administering a therapeutically effective dose of a pharmaceutical composition comprising a protease inhibitor of the present invention together with a pharmaceutically acceptable sterile vehicle.

Another aspect of this invention provides a method for inhibiting the activity of serine proteases of interest in a mammal comprising administering a therapeutically effective dose of a pharmaceutical composition comprising a protease inhibitor of the present invention together with a pharmaceutically acceptable sterile vehicle, wherein said serine proteases

are selected from the group consisting of: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; 5 proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

A further aspect of this invention relates to protease inhibitors comprising the following amino acid sequences: 10

X¹-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-Pro-Cys-Arg-Ala-X²-X³-X⁴-Arg-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-X⁵-Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-15 Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein X¹ is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu; X² is selected from Ala, Leu, Gly, or Met; X³ is selected from Ile, His, Leu, Lys, Ala, or Phe; X⁴ is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu; X⁵ is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr; provided that when X⁵ is Arg, X² is Ala or Leu; when X⁵ is Tyr, X² is Ala or X³ is His; and either X³ is not Ile; or X⁴ is not Ser; or X⁵ is not Leu, Phe, Met, Tyr, or Asn. Another aspect of this invention provides a protease inhibitor as defined above wherein X¹ 25 is Glu, X² is Met, X³ is Ile, X⁴ is Ile, and X⁵ is Gly.

The invention also relates more specifically to protease inhibitors comprising the following amino acid sequences:

30 Glu-Val-Val-Arg-Glu-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-Pro-Cys-Arg-Ala-X¹-X²-X³-Arg-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-X⁴-Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

35 wherein X¹ is selected from Ala, Leu, Gly, or Met; X² is selected from Ile, His, Leu, Lys, Ala, or Phe; X³ is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu; X⁴ is selected from Gly, Arg, Leu, Met, or Tyr; provided that when X¹ is Ala, X² is Ile, His, or

Leu; when X¹ is Leu, X² is Ile or His; when X¹ is Leu and X² is Ile, X³ is not Ser; when X¹ is Gly, X² is Ile; when X⁴ is Arg, X¹ is Ala or Leu; when X⁴ is Tyr, X¹ is Ala or X² is His; and either X¹ is not Met, or X² is not Ile, or X³ is not Ser, or X⁴ is not Gly.

A further aspect of this invention provides a protease inhibitor as defined above wherein X¹ is Met, X³ is Ser, and X⁴ is Gly. Another aspect of this invention provides a protease inhibitor wherein X² is selected from His, Ala, Phe, Lys, and Leu. Another aspect of this invention provides a protease inhibitor wherein X² is His. Another aspect of this invention provides a protease inhibitor wherein X² is Ala. Another aspect of this invention provides a protease inhibitor wherein X² is Phe. Another aspect of this invention provides a protease inhibitor wherein X² is Lys. Another aspect of this invention provides a protease inhibitor wherein X² is Leu. Another aspect of this invention provides a protease inhibitor wherein X¹ is Met, X² is Ile, and X⁴ is Gly.

Yet another aspect of this invention provides a protease inhibitor wherein X³ is Ile. Another aspect of this invention provides a protease inhibitor wherein X³ is Pro. Another aspect of this invention provides a protease inhibitor wherein X³ is Phe. Another aspect of this invention provides a protease inhibitor wherein X³ is Tyr. Another aspect of this invention provides a protease inhibitor wherein X³ is Trp. Another aspect of this invention provides a protease inhibitor wherein X³ is Asn. Another aspect of this invention provides a protease inhibitor wherein X³ is Leu.

An additional aspect of this invention provides a protease inhibitor wherein X³ is Lys. Another aspect of this invention provides a protease inhibitor wherein X³ is His. Another aspect of this invention provides a protease inhibitor wherein X³ is Glu. Another aspect of this invention provides a protease inhibitor wherein X¹ is Ala. Another aspect of this invention provides a

protease inhibitor wherein X^2 is Ile. Another aspect of this invention provides a protease inhibitor wherein X^3 is Phe, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^3 is Tyr, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^3 is Trp, and X^4 is Gly.

Yet another other aspect of this invention provides a protease inhibitor wherein X^3 is Ser or Phe, and X^4 is Arg or Tyr. Another aspect of this invention provides a protease inhibitor wherein X^2 is His or Leu, X^3 is Phe, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^1 is Leu. Another aspect of this invention provides a protease inhibitor wherein X^2 is His, X^3 is Asn or Phe, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^2 is Ile, X^3 is Pro, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^1 is Gly, X^2 is Ile, X^3 is Tyr, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^1 is Met, X^2 is His, X^3 is Ser, and X^4 is Tyr.

Additionally, another aspect of this invention relates to protease inhibitors comprising the following amino acid sequences:

X^1 -Val-Cys-Ser-Glu-Gln-Ala-Glu- X^2 -Gly-Pro-Cys-Arg-Ala- X^3 - X^4 - X^5 - X^6 -Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys- X^7 -Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein X^1 is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu; X^2 is selected from Thr, Val, Ile and Ser; X^3 is selected from Arg, Ala, Leu, Gly, or Met; X^4 is selected from Ile, His, Leu, Lys, Ala, or Phe; X^5 is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu; X^6 is selected from Arg, His, or Ala; and X^7 is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr.

Another aspect of this invention provides a protease inhibitor as defined above wherein at least two amino acid residues selected from the group consisting of X^3 ,

X⁴, X⁵, and X⁶ differ from the residues found in the naturally occurring sequence of KPI. Another aspect of this invention provides a protease inhibitor wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, Val, or Ser, X³ is Ala or Leu, X⁴ is Ile, X⁵ is Tyr, X⁶ is His and X⁷ is Gly. Another aspect of this invention provides a protease inhibitor wherein X² is Thr, and X³ is Ala. Another aspect of this invention provides a protease inhibitor wherein X² is Thr, and X³ is Leu. Another aspect of this invention provides a protease inhibitor wherein X² is Val, and X³ is Ala. Another aspect of this invention provides a protease inhibitor wherein X² is Ser, and X³ is Ala. Another aspect of this invention provides a protease inhibitor wherein X² is Val, and X³ is Leu. Another aspect of this invention provides a protease inhibitor wherein X² is Ser, and X³ is Leu. Another aspect of this invention provides a protease inhibitor wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Leu, X⁴ is Phe, X⁵ is Lys, X⁶ is Arg and X⁷ is Gly. Another aspect of this invention provides a protease inhibitor wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Leu, X⁴ is Phe, X⁵ is Lys, X⁶ is Arg and X⁷ is Tyr. Another aspect of this invention provides a protease inhibitor wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Leu, X⁴ is Phe, X⁵ is Lys, X⁶ is Arg and X⁷ is Leu.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

Figure 1 shows the strategy for the construction of plasmid pTW10:KPI.

Figure 2 shows the sequence of the synthetic gene for KPI (1→57) fused to the bacterial *phoA* secretory signal sequence.

5 Figure 3 shows the strategy for construction of plasmid pKPI-61.

Figure 4 shows the 192 bp *XbaI-HindIII* synthetic gene fragment encoding KPI (1→57) and four amino acids from yeast alpha-mating factor.

10 Figure 5 shows the synthetic 201 bp *XbaI-HindIII* fragment encoding KPI(-4→57) in PKPI-61.

Figure 6 shows the strategy for the construction of plasmid pTW113.

Figure 7 shows plasmid PTW113, encoding the 445 bp synthetic gene for yeast alpha-factor-KPI(-4→57) fusion.

15 Figure 8 shows the amino acid sequence for KPI (-4→57).

Figure 9 shows the strategy for constructing plasmid pTW6165.

20 Figure 10 shows plasmid, PTW6165, encoding the 445 bp synthetic gene for alpha-factor-KPI(-4→57; M15A, S17W) fusion.

25 Figure 11 shows the sequences of the annealed oligonucleotide pairs used to construct plasmids PTW6165, pTW6166, pTW6175, pBG028, pTW6183, pTW6184, pTW6185, pTW6173, and pTW6174.

Figure 12 shows the sequence of plasmid PTW6166 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15A, S17Y).

30 Figure 13 shows the sequence of plasmid PTW6175 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15L, S17F).

Figure 14 shows the sequence of plasmid pBG028 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15L, S17Y).

35 Figure 15 shows the sequence of plasmid PTW6183 encoding the fusion of yeast alpha-factor and KPI(-4→57; I16H, S17F).

Figure 16 shows the sequence of plasmid PTW6184 encoding the fusion of yeast alpha-factor and KPI(-4→57; I16H, S17Y).

5 Figure 17 shows the sequence of plasmid PTW6185 encoding the fusion of yeast alpha-factor and KPI(-4→57; I16H, S17W).

Figure 18 shows the sequence of plasmid PTW6173 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15A, I16H).

10 Figure 19 shows the sequence of plasmid PTW6174 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15L, I16H).

Figure 20 shows the amino acid sequence of KPI (-4→57; M15A, S17W).

15 Figure 21 shows the amino acid sequence of KPI (-4→57; M15A, S17Y).

Figure 22 shows the amino acid sequence of KPI (-4→57; M15L, S17F).

20 Figure 23 shows the amino acid sequence of KPI (-4→57; M15L, S17Y).

Figure 24 shows the amino acid sequence of KPI (-4→57; I16H, S17F).

Figure 25 shows the amino acid sequence of KPI (-4→57; I16H, S17Y).

25 Figure 26 shows the amino acid sequence of KPI (-4→57; I16H, S17W).

Figure 27 shows the amino acid sequence of KPI (-4→57; M15A, S17F).

30 Figure 28 shows the amino acid sequence of KPI (-4→57; M15A, I16H).

Figure 29 shows the amino acid sequence of KPI (-4→57; M15L, I16H).

Figure 30 shows the construction of plasmid pSP26:Amp:F1.

35 Figure 31 shows the construction of plasmid pgIII.

Figure 32 shows the construction of plasmid pPhoA:KPI:gIII.

Figure 33 shows the construction of plasmid pLG1.

Figure 34 shows the construction of plasmid pAL51.

Figure 35 shows the construction of plasmid pAL53.

Figure 36 shows the construction of plasmid PSP26:Amp:F1:PhoA:KPI:gIII.

Figure 37 shows the construction of plasmid pDW1 #14.

5 Figure 38 shows the coding region for the fusion of *phoA*-KPI (1→55)-geneIII.

Figure 39 shows the construction of plasmid PDW1 14-2.

10 Figure 40 shows the construction of KPI Library 16-19.

Figure 41 shows the expression unit encoded by the members of KPI Library 16-19.

15 Figure 42 shows the *phoA*-KPI(1→55)-geneIII region encoded by the most frequently occurring randomized KPI region.

Figure 43 shows the construction of pDD185 KPI (-4→57; M15A, S17F).

Figure 44 shows the sequence of alpha-factor fused to KPI (-4→57; M15A, S17F).

20 Figure 45 shows the inhibition constants (K_s) determined for purified KPI variants against the selected serine proteases kallikrein, factor Xa, and factor XIIa.

25 Figure 46 shows the inhibition constants (K_s) determined for KPI variants against kallikrein, plasmin, and factors Xa, XIa, and XIIa.

Figure 47 shows the post-surgical blood loss in pigs in the presence (KPI) and absence (NS) of KPI 185-1 (M15A, S17F).

30 Figure 48 shows the post-surgical hemoglobin loss in pigs in the presence (KPI) and absence (NS) of KPI 185-1 (M15A, S17F).

Figure 49 shows the oxygen tension in the presence and absence of KPI, before CPB, immediately after CPB, and at 60 and 180 minutes after the end of CPB.

35 Figure 50 summarizes the results shown in Figures 47-49.

Figure 51 shows the sequence of plasmid PTW6166 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15A, S17Y).

Figure 52 shows the sequence of plasmid PTW6175 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15L, S17F).

5 Figure 53 shows the sequence of plasmid PBG028 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15L, S17Y).

Figure 54 shows the inhibition constants (K_s) determined for KPI variants against kallikrein, plasmin, and factor XIIa.

10 Detailed Description

The present invention provides peptides that can bind to and preferably inhibit the activity of serine proteases. These inhibitory peptides can also provide a means of ameliorating, treating or preventing clinical
15 conditions associated with increased activity of serine proteases. The novel peptides of the present invention preferably exhibit a more potent and specific (i.e., greater) inhibitory effect toward serine proteases of interest than known serine protease inhibitors. Examples
20 of such proteases include: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants; particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3;
25 enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

Peptides of the present invention may be used to reduce the tissue damage caused by activation of the proteases of the contact pathway of the blood during
30 surgical procedures such as cardiopulmonary bypass (CPB). Inhibition of contact pathway proteases reduces the "whole body inflammatory response" that can accompany contact pathway activation, and that can lead to tissue damage, and possibly death. The peptides of the present
35 invention may also be used in conjunction with surgical procedures to reduce activated serine protease-associated perioperative and postoperative blood loss. For instance, perioperative blood loss of this type may be

particularly severe during CPB surgery. Pharmaceutical compositions comprising the peptides of the present invention may be used in conjunction with surgery such as CPB; administration of such compositions may occur preoperatively, perioperatively or postoperatively. Examples of other clinical conditions associated with increased serine protease activity for which the peptides of the present invention may be used include: CPB-induced inflammatory response; post-CPB pulmonary injury; pancreatitis; allergy-induced protease release; deep vein thrombosis; thrombocytopenia; rheumatoid arthritis; adult respiratory distress syndrome; chronic inflammatory bowel disease; psoriasis; hyperfibrinolytic hemorrhage; organ preservation; wound healing; and myocardial infarction. Other examples of preferable uses of the peptides of the present invention are described in U.S. Patent No. 5,187,153.

The invention is based upon the novel substitution of amino acid residues in the peptide corresponding to the naturally occurring KPI protease inhibitor domain of human amyloid β -amyloid precursor protein (APPI). These substitutions produce peptides that can bind to serine proteases and preferably exhibit an inhibition of the activity of serine proteases. The peptides also preferably exhibit a more potent and specific serine protease inhibition than known serine protease inhibitors. In accordance with the invention, peptides are provided that may exhibit a more potent and specific inhibition of one or more serine proteases of interest, e.g., kallikrein, plasmin and factors Xa, XIa, XIIa, and XIIIa.

The present invention also includes pharmaceutical compositions comprising an effective amount of at least one of the peptides of the invention, in combination with a pharmaceutically acceptable sterile vehicle, as described in REMINGTON'S PHARMACEUTICAL SCIENCES: DRUG RECEPTORS AND RECEPTOR THEORY, (18th ed.), Mack Publishing Co., Easton, PA (1990).

A. Selection of sequences of KPI variants

The sequence of KPI is shown in Table 1. Table 2 shows a comparison of this sequence with that of aprotinin, with which it shares about 45% sequence identity. The numbering convention for KPI shown in Table 1 and used hereinafter designates the first glutamic acid residue of KPI as residue 1. This corresponds to residue number 3 using the standard numbering convention for aprotinin.

The crystal structure for KPI complexed with trypsin has been determined. See Perona et al., *J. Mol. Biol.* 230:919 (1993). The three-dimensional structure reveals two binding loops within KPI that contact the protease. The first loop extends from residue Thr⁹ to Ile¹⁶, and the second loop extends from residue Phe³² to Gly³⁷. The two protease binding loops are joined through the disulfide bridge extending from Cys¹² to Cys³⁶. KPI contains two other disulfide bridges, between Cys³ and Cys⁵³, and between Cys²⁸ to Cys⁴⁹.

This structure was used as a guide to inform our strategy for making the amino acid residue substitutions that will be most likely to affect the protease inhibitory properties of KPI. Our examination of the structure indicated that certain amino acid residues, including residues 9, 11, 13-18, 32, and 37-40, appear to be of particular significance in determining the protease binding properties of the KPI peptide. In a preferred embodiment of the invention two or more of those KPI peptide residues are substituted; such substitutions preferably occurring among residues 9, 11, 13-18, 32, and 37-40. In particular, we found that those substituted peptides, including peptides comprising substitutions of at least two of the four residues at positions 15-18, may exhibit more potent and specific serine protease inhibition toward selected serine proteases of interest than exhibited by the natural KPI peptide domain. Such substituted peptides may further comprise one or more additional substitutions at residues 9, 11, 13, 14, 32 and 37-40; in particular, such peptides may further

comprise a substitution at positions 9 or 37, or an additional substitution at residue 13. In particular, the peptides of the present invention preferably exhibit a greater potency and specificity for inhibiting one or more serine proteases of interest (e.g., kallikrein, plasmin and factors VIIa, IXa, Xa, XIa, and XIIa) than the potency and specificity exhibited by native KPI or other known serine protease inhibitors. That greater potency and specificity may be manifested by the peptides of the present invention by exhibiting binding constants for serine proteases of interest that are less than the binding constants exhibited by native KPI, or other known serine protease inhibitors, for such proteases.

As an initial guide to informing the choices of amino acid substitution for preparation of KPI variants, the sequences and protease inhibitory activities of aprotinin and KPI are compared. Aprotinin is twice as potent as wild-type KPI with respect to inhibition of human plasma kallikrein, and is 100-fold more potent as an inhibitor of human plasmin. There are three amino acid differences between aprotinin and wild-type KPI in the first protease binding loop extending from residues 9 to 17. A series of KPI variants may then be created, using the methods detailed below, where the residues present in aprotinin at positions 13, 15 and 17 are substituted with the residues found in KPI. The effect of such substitutions upon KPI inhibition of plasma kallikrein and plasmin is then determined.

These results show that substitution of arginine at position 13 by lysine significantly reduces the activity of the resulting protein as an inhibitor of plasma kallikrein. Similarly, substituting positions 15 and 17 of KPI with the corresponding residues found in aprotinin also decreases potency of the KPI variants against kallikrein. Substitutions of aprotinin residues at positions 13 and 15, however, increase the potency of KPI toward plasmin. The single change of methionine to arginine at position 15 (designated M15R) decreases the K_i against plasmin more than 10-fold. The change of

serine to isoleucine at position 17 (S17I) decreases the potency of KPI toward plasmin.

It is observed that single-amino acid substitutions in the first protease binding loop are generally additive, that is, combinations of single amino-acid substitutions, each of which individually enhance the potency toward plasmin, result in variants with even higher potency. The substitution R13K results in a plasmin K_i of 12.3, and the further exchange of M15R results in a K_i that is reduced to 1.45.

It appears, therefore, from these results that combinations of favorable single amino acid substitutions can result in enhanced potency of KPI variants. It is further apparent that substitution in KPI with the residues found in the aprotinin first protease binding loop is not always useful. Although aprotinin is a more potent kallikrein inhibitor than KPI, none of the combinations of aprotinin residues in KPI improve kallikrein inhibition.

To further investigate substitutions that might usefully enhance protease inhibition, a series of single substitutions in KPI is prepared where charged residues in the first protease binding loop are systematically replaced with alanine. This is intended to determine whether substitutions at these sites affect potency toward plasma kallikrein, factor XIIa or plasmin.

It is found that replacement of arginine at position 13 (R13A) drastically reduces KPI inhibition of kallikrein, XIIa or plasmin. The replacement I16A, however, significantly increases the K_i towards both kallikrein and plasmin, suggesting that this amino acid position is critical to inhibition of these proteases. The S17A substitution has little effect. The substitution R18A has little effect upon plasmin inhibition, but significantly impacts inhibition of kallikrein and factor XIIa. These results suggest that substitutions at positions I16 and R18 have the potential to significantly alter the potency of KPI toward kallikrein or plasmin.

These results also suggest that substitutions at residues M15 and S17 could have major effects upon inhibition of kallikrein, XIIa or plasmin. To investigate this further, two sets of yeast expression
5 plasmids are prepared, using the methods described in detail below, in which either M15 or S17 are replaced with all possible amino acids.

Yeast are transformed with these two sets of plasmids, and 100 individual colonies are picked at
10 random from each transformation. Small cultures are grown from each of these colonies, and their conditioned broth is harvested and tested for kallikrein inhibiting activity. The plasmids from colonies yielding cultures expressing KPI variants more potent than wild-type KPI
15 are isolated, and the KPI domain are sequenced. It is found that only four 4 substitutions at position 15: M15A,M15L,M15S,M15V; and 4 substitutions at position 17: S17P,S17F,S17Y and S17W, result in KPI variants with improved potency toward kallikrein.

20 Combinations of these position 15 and 17 mutants are then prepared to test if their effects on potency of protease inhibition are additive. Four of these double mutants ([M15A,S17Y], [M15A,S17W], [M15L,S17Y] and [M15L,S17F]) are substantially more potent toward
25 kallikrein and factor XIIa than the single amino acid substitutions on which they are based.

The results of changing arginine at positions 18 for alanine also suggest that substitutions at position 18 could affect inhibition of kallikrein and factor XIIa.
30 The KPI double variant M13A,S17W (named TW6165 below) is used to construct a series of variants where all possible amino acid substitutions other than Cys and Arg are placed at position 18. Of these variants, three ([M13A,S17W, R18H], [M13A,S17W, R18Q], and [M13A,S17W,
35 R18T]) are found to exhibit enhanced inhibition of kallikrein and Factor XIIa.

The results described above relate to proteins having the N-terminal sequence EVVREVCN- et seq., as found in KPI (-4-57). The present invention also relates, however

to proteins wherein the N-terminal sequence may be varied, preferably by substituting aspartic acid at the N-terminus in place of the glutamic acid (i.e. the N-terminal sequence is DVVREVCs-). Other N-terminal sequences that may be used will be apparent to the skilled artisan, including a sequence lacking the first four amino acids of KPI(-4→57), i.e. having the sequence EVCS-.

By way of example, and as set forth in greater detail below, the serine protease inhibitory properties of peptides of the present invention were measured for the serine proteases of interest — kallikrein, plasmin and factors Xa, XIa, and XIIa. Methodologies for measuring the inhibitory properties of the KPI variants of the present invention are known to those skilled in the art, e.g., by determining the inhibition constants of the variants toward serine proteases of interest, as described in Example 4, *infra*. Such studies measure the ability of the novel peptides of the present invention to bind to one or more serine proteases of interest and to preferably exhibit a greater potency and specificity for inhibiting one or more serine protease of interest than known serine protease inhibitors such as native KPI.

The ability of the peptides of the present invention to bind one or more serine proteases of interest, particularly the ability of the peptides to exhibit such greater potency and specificity toward serine proteases of interest, manifest the clinical and therapeutic applications of such peptides. The clinical and therapeutic efficacy of the peptides of the present invention can be assayed by *in vitro* and *in vivo* methodologies known to those skilled in the art, e.g., as described in Example 5, *infra*.

Table 1: SEQUENCE OF KPI:

| | | | |
|---|---|----|----|
| | 10 | 20 | 30 |
| 1 | V R E V C S E Q A E T G P C R A M I S R W Y F D V T E G K C A P | | |
| | 40 | 50 | |
| | F F Y G G C G G N R N N F D T E E Y C M A V C G S A I | | |

Table 2: COMPARISON OF KPI AND APROTININ SEQUENCES:

| | | | | | |
|-------|---|----|----|----|----|
| | 10 | 20 | 30 | 40 | 50 |
| KPI: | V R E V C S E Q A E T G P C R A M I S R W Y F D V T E G K C A P F F Y G G C G G N R N N F D T E E Y C M A V C G S A I | | | | |
| BPTI: | R P D F C L E P P Y T G P C K A R I I R Y F Y N A K A G L C Q T F Y G G C R A K R N N F K S A E D C M R T C G G A | | | | |
| | 1 | 10 | 20 | 30 | 40 |
| | | | | | 50 |

B. Methods of producing KPI variants

The peptides of the present invention can be created by synthetic techniques or recombinant techniques which employ genomic or cDNA cloning methods.

5 1. Production by chemical synthesis

Peptides of the present invention can be routinely synthesized using solid phase or solution phase peptide synthesis. Methods of preparing relatively short peptides such as KPI by chemical synthesis are well known
10 in the art. KPI variants could, for example be produced by solid-phase peptide synthesis techniques using commercially available equipment and reagents such as those available from Milligen (Bedford, MA) or Applied Biosystems-Perkin Elmer (Foster City, CA).
15 Alternatively, segments of KPI variants could be prepared by solid-phase synthesis and linked together using segment condensation methods such as those described by Dawson et al., *Science* 266:776 (1994). During chemical
20 synthesis of the KPI variants, substitution of any amino acid is achieved simply by replacement of the residue that is to be substituted with a different amino acid monomer.

2. Production by recombinant DNA technology

25 (a) Preparation of genes encoding KPI variants

In a preferred embodiment of the invention, KPI variants are produced by recombinant DNA technology. This requires the preparation of genes encoding each KPI variant that is to be made. Suitable genes can be
30 constructed by oligonucleotide synthesis using commercially available equipment, such as that provided by Milligen and Applied Biosystems, *supra*. The genes can be prepared by synthesizing the entire coding and non-coding strands, followed by annealing the two strands.
35 Alternatively, the genes can be prepared by ligation of smaller synthetic oligonucleotides by methods well known in the art. Genes encoding KPI variants are produced by

varying the nucleotides introduced at any step of the synthesis to change the amino acid sequence encoded by the gene.

5 Preferably, however, KPI variants are made by site-directed mutagenesis of a gene encoding KPI. Methods of site-directed mutagenesis are well known in the art. See, for example, Ausubel et al., (eds.) CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Wiley Interscience, 1987); PROTEIN ENGINEERING (Oxender & Fox eds., A. Liss, Inc. 1987). These methods require the availability of a gene encoding KPI or a variant thereof, which can then be mutagenized by known methods to produce the desired KPI variants. In addition, linker-scanning and polymerase chain reaction ("PCR") mediated techniques can be used for purposes of mutagenesis. See PCR TECHNOLOGY (Erlich ed., Stockton Press 1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, vols. 1 & 2, loc. cit.

20 A gene encoding KPI can be obtained by cloning the naturally occurring gene, as described for example in U.S. Patents Nos. 5,223,482 and 5,187,153, which are hereby incorporated by reference in their entireties. In particular, see columns 6-9 of U.S. Patent No. 5,187,153. See also PCT Application No. 93/09233. In a preferred embodiment of the invention a synthetic gene encoding KPI is produced by chemical synthesis, as described above. The gene may encode the 57-amino acid KPI domain shown in Table 1, or it may also encode additional N-terminal amino acids from the APPI protein sequence, such as the four amino acid sequence (Glu-Val-Val-Arg, designated residues -4 to -1) immediately preceding the KPI domain in APPI.

35 Production of the gene by synthesis allows the codon usage of the KPI gene to be altered to introduce convenient restriction endonuclease recognition sites, without altering the sequence of the encoded peptide. In a preferred embodiment of the invention, the synthetic KPI gene contains restriction endonuclease recognition sites that facilitate excision of DNA cassettes from the KPI gene. These cassettes can be replaced with small

synthetic oligonucleotides encoding the desired changes in the KPI peptide sequence. See Ausubel, *supra*.

This method also allows the production of genes encoding KPI as a fusion peptide with one or more additional peptide or protein sequences. The DNA encoding these additional sequences is arranged in-frame with the sequence encoding KPI such that, upon translation of the gene, a fusion protein of KPI and the additional peptide or protein sequence is produced. Methods of making such fusion proteins are well known in the art. Examples of additional peptide sequences that can be encoded in the genes are secretory signal peptide sequences, such as bacterial leader sequences, for example *ompA* and *phoA*, that direct secretion of proteins to the bacterial periplasmic space. In a preferred embodiment of the invention, the additional peptide sequence is a yeast secretory signal sequence, such as α -mating factor, that directs secretion of the peptide when produced in yeast.

Additional genetic regulatory sequences can also be introduced into the synthetic gene that are operably linked to the coding sequence of the gene, thereby allowing synthesis of the protein encoded by the gene when the gene is introduced into a host cell. Examples of regulatory genetic sequences that can be introduced are: promoter and enhancer sequences and transcriptional and translational control sequences. Other regulatory sequences are well known in the art. See Ausubel et al., *supra*, and Sambrook et al., *supra*.

Sequences encoding other fusion proteins and genetic elements are well known to those of skill in the art. In a preferred embodiment of the invention, the KPI sequence is prepared by ligating together synthetic oligonucleotides to produce a gene encoding an in-frame fusion protein of yeast α -mating factor with either KPI (1-57) or KPI (-4-57).

The gene constructs prepared as described above are conveniently manipulated in host cells using methods of manipulating recombinant DNA techniques that are well

known in the art. See, for example Sambrook et al.,
MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition,
(Cold Spring Harbor Laboratory Press, Cold Spring Harbor,
NY 1989), and Ausubel, *supra*. In a preferred embodiment
5 of the invention the host cell used for manipulating the
KPI constructs is *E. coli*. For example, the construct
can be ligated into a cloning vector and propagated in *E.*
coli by methods that are well known in the art. Suitable
cloning vectors are described in Sambrook, *supra*, or are
10 commercially available from suppliers such as Promega
(Madison, WI), Stratagene (San Diego, CA) and Life
Technologies (Gaithersburg, MD).

Once a gene construct encoding KPI has been obtained,
genes encoding KPI variants are obtained by manipulating
15 the coding sequence of the construct by standard methods
of site-directed mutagenesis, such as excision and
replacement of small DNA cassettes, as described *supra*.
See Ausubel, *supra*, and Sinha et al., *supra*. See also
U.S. Patent 5,373,090, which is herein incorporated by
20 reference in its entirety. See particularly, columns
4-12 of U.S. Patent 5,272,090. These genes are then used
to produce the KPI variant peptides as described below.

Alternatively, KPI variants can be produced using
phage display methods. See, for example, Dennis et al.
25 *supra*, which is hereby incorporated by reference in its
entirety. See also U.S. Patent Nos. 5,223,409 and
5,403,484, which are hereby also incorporated by
reference in their entireties. In these methods,
libraries of genes encoding variants of KPI are fused in-
30 frame to genes encoding surface proteins of filamentous
phage, and the resulting peptides are expressed
(displayed) on the surface of the phage. The phage are
then screened for the ability to bind, under appropriate
conditions, to serine proteases of interest immobilized
35 on a solid support. Large libraries of phage can be
used, allowing simultaneous screening of the binding
properties of a large number of KPI variants. Phage that
have desirable binding properties are isolated and the
sequences of the genes encoding the corresponding KPI

variants is determined. These genes are then used to produce the KPI variant peptides as described below.

(b) *Expression of KPI variant peptides*

5 Once genes encoding KPI variants have been prepared, they are inserted into an expression vector and used to produce the recombinant peptide. Suitable expression vectors and corresponding methods of expressing recombinant proteins and peptides are well known in the
10 art. Methods of expressing KPI peptides are described in U.S. Patent 5,187,153, columns 9-11, U.S. Patent 5,223,482, columns 9-11, and PCT application 93/09233, pp. 49-67. See also Ausubel et al., *supra*, and Sambrook et al., *supra*. The gene can be expressed in any number
15 of different recombinant DNA expression systems to generate large amounts of the KPI variant, which can then be purified and tested for its ability to bind to and inhibit serine proteases of interest.

 Examples of expression systems known to the skilled
20 practitioner in the art include bacteria such as *E. coli*, yeast such as *Saccharomyces cerevisiae* and *Pichia pastoris*, baculovirus, and mammalian expression systems such as in Cos or CHO cells. In a preferred embodiment, KPI variants are expressed in *Pichia pastoris*. In
25 another preferred embodiment the KPI variants are cloned into expression vectors to produce a chimeric gene encoding a fusion protein of the KPI variant with yeast α -mating factor. The mating factor acts as a signal sequence to direct secretion of the fusion protein from
30 the yeast cell, and is then cleaved from the fusion protein by a membrane-bound protease during the secretion process. The expression vector is transformed into *S. cerevisiae*, the transformed yeast cells are cultured by standard methods, and the KPI variant is purified from
35 the yeast growth medium.

 Recombinant bacterial cells expressing the peptides of the present invention, for example, *E. coli*, are grown in any of a number of suitable media, for example LB, and the expression of the recombinant antigen induced by

adding IPTG to the media or switching incubation to a higher temperature. After culturing the bacteria for a further period of between 2 and 24 hours, the cells are collected by centrifugation and washed to remove residual media. The bacterial cells are then lysed, for example, by disruption in a cell homogenizer and centrifuged to separate dense inclusion bodies and cell membranes from the soluble cell components. This centrifugation can be performed under conditions whereby dense inclusion bodies are selectively enriched by incorporation of sugars such as sucrose into the buffer and centrifugation at a selective speed. If the recombinant peptide is expressed in inclusion bodies, as is the case in many instances, these can be washed in any of several solutions to assist in the removal of any contaminating host proteins, then solubilized in solutions containing high concentrations of urea (e.g., 8M) or chaotropic agents such as guanidine hydrochloride in the presence of reducing agents such as β -mercaptoethanol or DTT (dithiothreitol).

At this stage it may be advantageous to incubate the peptides of the present invention for several hours under conditions suitable for the peptides to undergo a refolding process into a conformation which more closely resembles that of native KPI. Such conditions generally include low protein concentrations less than 500 μ g/ml, low levels of reducing agent, concentrations of urea less than 2M and often the presence of reagents such as a mixture of reduced and oxidized glutathione which facilitate the interchange of disulphide bonds within the protein molecule. The refolding process can be monitored, for example, by SDS-PAGE or with antibodies which are specific for the native molecule (which can be obtained from animals vaccinated with the native molecule isolated from parasites). Following refolding, the peptide can then be purified further and separated from the refolding mixture by chromatography on any of several supports including ion exchange resins, gel permeation resins or on a variety of affinity columns.

Purification of KPI variants can be achieved by standard methods of protein purification, e.g., using various chromatographic methods including high performance liquid chromatography and adsorption chromatography. The purity and the quality of the peptides can be confirmed by amino acid analyses, molecular weight determination, sequence determination and mass spectrometry. See, for example, PROTEIN PURIFICATION METHODS — A PRACTICAL APPROACH, Harris et al., eds. (IRL Press, Oxford, 1989). In a preferred embodiment, the yeast cells are removed from the growth medium by filtration or centrifugation, and the KPI variant is purified by affinity chromatography on a column of trypsin-agarose, followed by reversed-phase HPLC.

C. Measurement of protease inhibitory properties of KPI variants

Once KPI variants have been purified, they are tested for their ability to bind to and inhibit serine proteases of interest in vitro. The peptides of the present invention preferably exhibit a more potent and specific inhibition of serine proteases of interest than known serine protease inhibitors, such as the natural KPI peptide domain. Such binding and inhibition can be assayed for by determining the inhibition constants for the peptides of the present invention toward serine proteases of interest and comparing those constants with constants determined for known serine protease inhibitors, e.g., the native KPI domain, toward those proteases. Methods for determining inhibition constants of protease inhibitors are well known in the art. See Fersht, ENZYME STRUCTURE AND MECHANISM, 2nd ed., W.H. Freeman and Co., New York, (1985).

In a preferred embodiment the inhibition experiments are carried out using a chromogenic synthetic protease substrate, as described, for example, in Bender et al., J. Amer. Chem. Soc. 88:5890 (1966). Measurements taken by this method can be used to calculate inhibition

constants (K_i values) of the peptides of the present invention toward serine proteases of interest. See Bieth in BAYER-SYMPOSIUM V "PROTEINASE INHIBITORS", Fritz et al., eds., pp. 463-69, Springer-Verlag, Berlin, Heidelberg, New York, (1974). KPI variants that exhibit potent and specific inhibition of one or more serine proteases of interest may subsequently be tested *in vivo*. *In vitro* testing, however, is not a prerequisite for *in vivo* studies of the peptides of the present invention.

10 **D. Testing of KPI variants *in vivo***

 The peptides of the present invention may be tested, alone or in combination, for their therapeutic efficacy by various *in vivo* methodologies known to those skilled in the art, e.g., the ability of KPI variants to reduce postoperative bleeding can be tested in standard animal models. For example, cardiopulmonary bypass surgery can be carried out on animals such as pigs in the presence of KPI variants, or in control animals where the KPI variant is not used. The use of pigs as a model for studying the clinical effects associated with CPB has previously been described. See Redmond et al., *Ann. Thorac. Surg.* 56:474 (1993).

25 The KPI variant is supplied to the animals in a pharmaceutical sterile vehicle by methods known in the art, for example by continuous intravenous infusion. Chest tubes can be used to collect shed blood for a defined period of time. The shed blood, together with the residual intrathoracic blood found after sacrifice of the animal can be used to calculate hemoglobin (Hgb) loss. The postoperative blood and Hgb loss is then compared between the test and control animals to determine the effect of the KPI variants.

E. Therapeutic use of KPI variants

35 KPI variants of the present invention found to exhibit therapeutic efficacy (e.g., reduction of blood loss following surgery in animal models) may preferably be used and administered, alone or in combination or as

a fusion protein, in a manner analogous to that currently used for aprotinin or other known serine protease inhibitors. See Butler et al., *supra*. Peptides of the present invention generally may be administered in the manner that natural peptides are administered. A therapeutically effective dose of the peptides of the present invention preferably affects the activity of the serine proteases of interest such that the clinical condition may be treated, ameliorated or prevented. Therapeutically effective dosages of the peptides of the present invention can be determined by those skilled in the art, e.g., through *in vivo* or *in vitro* models. Generally, the peptides of the present invention may be administered in total amounts of approximately 0.01 to approximately 500, specifically 0.1 to 100 mg/kg body weight, if desired in the form of one or more administrations, to achieve therapeutic effect. It may, however, be necessary to deviate from such administration amounts, in particular depending on the nature and body weight of the individual to be treated, the nature of the medical condition to be treated, the type of preparation and the administration of the peptide, and the time interval over which such administration occurs. Thus, it may in some cases be sufficient to use less than the above amount of the peptides of the present invention, while in other cases the above amount is preferably exceeded. The optimal dose required in each case and the type of administration of the peptides of the present invention can be determined by one skilled in the art in view of the circumstances surrounding such administration. Such peptides can be administered by intravenous injections, *in situ* injections, local applications, inhalation, oral administration using coated polymers, dermal patches or other appropriate means. Compositions comprising peptides of the present invention are advantageously administered in the form of injectable compositions. Such peptides may be preferably administered to patients via continuous intravenous infusion, but can also be administered by single or

multiple injections. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, 5 preservatives, buffers and the like, as described in REMINGTON'S PHARMACEUTICAL SCIENCES, pp. 1405-12 and 1461-87 (1975) and THE NATIONAL FORMULARY XIV., 14th Ed. Washington: American Pharmaceutical Association (1975). Aqueous carriers include water, alcoholic/aqueous 10 solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, etc. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobials, anti-oxidants, chelating agents and inert gases. The pH and exact 15 concentration of the various components of the composition are adjusted according to routine skills in the art. See GOODMAN AND GILMAN'S THE PHARMACOLOGICAL BASIS FOR THERAPEUTICS (7th ed.). The peptides of the present invention may be present in such pharmaceutical 20 preparations in a concentration of approximately 0.1 to 99.5% by weight, specifically 0.5 to 95% by weight, relative to the total mixture. Such pharmaceutical preparations may also comprise other pharmaceutically active substances in addition to the peptides of the 25 present invention. Other methods of delivering the peptides to patients will be readily apparent to the skilled artisan.

Examples of mammalian serine proteases that may exhibit inhibition by the peptides of the present 30 invention include: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as thrombin and factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; 35 proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator. Examples of conditions associated with increased serine protease activity include: CPB-induced inflammatory response; post-CPB pulmonary injury; pancreatitis; allergy-induced

protease release; deep vein thrombosis; thrombocytopenia; rheumatoid arthritis; adult respiratory distress syndrome; chronic inflammatory bowel disease; psoriasis; hyperfibrinolytic hemorrhage; organ preservation; wound healing; and myocardial infarction. Other examples of the use of the peptides of the present invention are described in U.S. Patent No. 5,187,153.

The inhibitors of the present invention may also be used for inhibition of serine protease activity *in vitro*, for example during the preparation of cellular extracts to prevent degradation of cellular proteins. For this purpose the inhibitors of the present invention may preferably be used in a manner analogous to the way that aprotinin, or other known serine protease inhibitors, are used. The use of aprotinin as a protease inhibitor for preparation of cellular extracts is well known in the art, and aprotinin is sold commercially for this purpose.

The present invention, thus generally described, will be understood more readily by reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention.

EXAMPLES

Example 1. Expression of wild-type KPI (-4-57)

A. Construction of PTW10:KPI

Plasmid PTW10:KPI is a bacterial expression vector encoding the 57 amino acid form of KPI fused to the bacterial *phoA* signal sequence. The strategy for the construction of PTW10:KPI is shown in Figure 1.

Plasmid pcDNAII (Invitrogen, San Diego, CA) was digested with *PvuII* and the larger of the two resulting *PvuII* fragments (3013 bp) was isolated. Bacterial expression plasmid pSP26 was digested with *MluI* and *RsrII*, and the 409 bp *MluI*-*RsrII* fragment containing the *pTrp* promoter element and transcription termination signals was isolated by electrophoresis in a 3% NuSieve Agarose gel (FMC Corp., Rockland, ME). Plasmid pSP26, containing a heparin-binding EGF-like growth factor (HB-EGF) insert between the *NdeI* and *HindIII* sites, is

described as pNA28 in Thompson et al., *J. Biol. Chem.* 269:2541 (1994). Plasmid pSP26 was deposited in host *E. coli* W3110, pSP26 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, 20852, USA under the conditions specified by the Budapest Treaty on the International Recognition of the Deposit of Microorganisms (Budapest Treaty). Host *E. coli* W3110, pSP26 was deposited on 3 May 1995 and given Accession No. 69800. Availability of the deposited plasmid is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The ends of the *Mlu*I-*Rsr*II fragment were blunted using DNA polymerase Klenow fragment by standard techniques. The blunted fragment of pSP26 was then ligated into the large *Pvu*II fragment of plasmid pCDNAII, and the ligation mixture was used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were selected and used to isolate plasmid pTW10 by standard techniques.

A synthetic gene was constructed encoding the bacterial *phoA* secretory signal sequence fused to the amino terminus of KPI(1-57). The synthetic gene contains cohesive ends for *Nde*I and *Hind*III, and also incorporates restriction endonuclease recognition sites for *Age*I, *Rsr*II, *Aat*II and *Bam*HI, as shown in Figure 2. The synthetic *phoA*-KPI gene was constructed from 6 oligonucleotides of the following sequences (shown 5'→3'):

6167:

TATGAAACAAAGCACTATTGCACTGGCACTCTTACCGTTACTGTTTACCC
CTGTGACAAAAGCCGAGGTGTGCTCTGAA

6169:

CTCGGCTTTTGTACAGGGGTAAACAGTAACGGTAAGAGTGCCAGTGCAA
TAGTGCTTTGTTTCATA

6165:

CAAGCTGAGACCGGTCCGTGCCGTGCAATGATCTCCGCTGGTACTTTGA
CGTCACTGAAGGTAAGTGCGCTCCATTCTTT

6166:
GCACCTTACCTTCAGTGACGTCAAAGTACCAGCGGGAGATCATTGCACGGC
ACGGACCGGTCTCAGCTTGTTTCAGAGCACAC

6168:
TACGGCGGTTGCGGCGGCAACCGTAACAACCTTTGACACTGAAGAGTACTG
CATGGCAGTGTGCGGATCCGCTATTTAAGCT

6164:
AGCTTAAATAGCGGATCCGCACACTGCCATGCAGTACTCTTCAGTGTCAA
AGTTGTTACGGTTGCCGCCGCAACCGCCGTAAAAGAATGGAGC

10 The oligonucleotides were phosphorylated and annealed
in pairs: 6167 + 6169, 6165 + 6166, 6168 + 6164. In
20 μ l T4 DNA Ligase Buffer (New England Biolabs,
Beverly, MA), 1 μ g of each oligonucleotide pair was
incubated with 10 U T4 Polynucleotide Kinase (New England
15 Biolabs) for 1 h at 37°C, then heated to 95°C for 1
minute, and slow-cooled to room temperature to allow
annealing. All three annealed oligo pairs were then
mixed for ligation to one another in a total volume of
100 μ l T4 DNA Ligase Buffer, and incubated with 400 U T4
20 DNA Ligase (New England Biolabs) overnight at 15°C. The
ligation mixture was extracted with an equal volume of
phenol:CHCl₃ (1:1), ethanol-precipitated, resuspended in
50 μ l Restriction Endonuclease Buffer #4 (New England
Biolabs) and digested with *Nde*I and *Hind*III. The
25 annealed, ligated and digested oligos were then subjected
to electrophoresis in a 3% NuSieve Agarose gel, and the
240 bp *Nde*I-*Hind*III fragment was excised. This gel-
purified synthetic gene was ligated into plasmid pTW10
which had previously been digested with *Nde*I and *Hind*III,
30 and the ligation mixture was used to transform *E. coli*
strain MC1061. Ampicillin-resistant colonies were
selected and used to prepare plasmid pTW10:KPI. This
plasmid contains the *phoA*-KPI(1-57) fusion protein
inserted between the pTrp promoter element and the
35 transcription termination signals.

B. Construction of pKPI-61

The strategy for constructing pKPI-61 is shown in
Figure 3. Plasmid pTW10:KPI was digested with *Age*I and

*Hind*III; the resulting 152 bp *Age*I-*Hind*III fragment containing a portion of the KPI synthetic gene was isolated by preparative gel electrophoresis. An oligonucleotide pair (129 + 130) encoding the 9 amino-terminal residues of KPI(1→57) and 4 amino acids of yeast α -mating factor was phosphorylated and annealed as described above.

129: CTAGATAAAAGAGAGGTGTGCTCTGAACAAGCTGAGA

130: CCGGTCTCAGCTTGTTTCAGAGCACACCTCTCTTTTAT

10 The annealed oligonucleotides were then ligated to the *Age*I-*Hind*III fragment of the KPI (1→57) synthetic gene. The resulting 192 bp *Xba*I-*Hind*III synthetic gene (shown in Figure 4) was purified by preparative gel electrophoresis, and ligated into plasmid pUC19 which had
15 previously been digested with *Xba*I and *Hind*III. The ligation products were used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were picked and used to prepare plasmid PKPI-57 by standard methods. To create a synthetic gene encoding KPI(-4→57), PKPI-57 was
20 digested with *Xba*I and *Age*I and the smaller fragment replaced with annealed oligos 234 + 235, which encode 4 amino acid residues of yeast α -mating factor fused a 4 amino acid residue amino-terminal extension of KPI(1→57).

234: CTAGATAAAAGAGAGGTGTGTTAGAGAGGTGTGCTCTGAACAAGCTGAGA

25 235: CCGGTCTCAGCTTGTTTCAGAGCACACCTCTCTAACAACCTCTCTTTTAT

The 4 extra amino acids are encoded in the amyloid β -protein precursor/protease nexin-2 (APPI) which contains the KPI domain. The synthetic 201 bp *Xba*I-*Hind*III fragment encoding KPI(-4→57) in pKPI-61 is shown
30 in Figure 5.

C. Assembly of pTW113

The strategy for the construction of PTW113 is shown in Figure 6. Plasmid pSP35 was constructed from yeast expression plasmid pYES2 (Invitrogen, San Diego, CA) as

follows. A 267 bp *Pvu*II-*Xba*I fragment was generated by PCR from yeast α -mating factor DNA using oligos 6274 and 6273:

6274: GGGGGCAGCTGTATAAACGATTAAAA
5 6273: GGGGGTCTAGAGATACCCCTTCTTCTTTAG

10 This PCR fragment, encoding an 82 amino acid portion of yeast α -mating factor, including the secretory signal peptide and pro-region, was inserted into pYES2 that had been previously digested with *Pvu*II and *Xba*I. The resulting plasmid is denoted pSP34.

Two oligonucleotide pairs, 6294 + 6292 were then ligated to 6290 + 6291, and the resulting 135 bp fragment was purified by gel electrophoresis.

6294: CTAGATAAAAGAGAGGCTGAGGCTCACGCTGAAGGTACTTTCACTTC
15 6290: TGACGTCTCTTCTTACTTGGAAGGTCAAGCTGCTAAGGAATTCAT
CGCTTGTTGGTCAAAGGTAGAGGTTAAGCTTA
6291: CTAGTAAGCTTAACCTCTACCTTTGACCAACCAAGCGATGAATTC
CTTAGCA
20 6292: GCTTGACCTTCCAAGTAAGAAGAGACGTCAGAAGTGAAAGTACCT
TCAGCGTGAGCCTCAGCCTCTCTTTTAT

25 The resulting synthetic fragment was ligated into the *Xba*I site of pSP34, resulting in plasmid pSP35. pSP35 was digested with *Xba*I and *Hind*III to remove the insert, and ligated with the 201 bp *Xba*I-*Hind*III fragment of pKPI-61, encoding KPI(-4 \rightarrow 57). The resulting plasmid pTW113, encodes the 445 bp synthetic gene for the α -factor-KPI(-4 \rightarrow 57) fusion. See Figure 7.

D. Transformation of yeast with pTW113

30 *Saccharomyces cerevisiae* strain ABL115 was transformed with plasmid pTW113 by electroporation by the method of Becker et al., *Methods Enzymol.* 194:182 (1991). An overnight culture of yeast strain ABL115 was used to inoculate 200 ml YPD medium. The inoculated culture was grown with vigorous shaking at 30°C to an OD₆₀₀ of 1.3-1.5,

at which time the cells were harvested by centrifugation at 5000 rpm for 5 minutes. The cell pellet was resuspended in 200 ml ice-cold water, respun, resuspended in 100 ml ice-cold water, then pelleted again. The washed cell pellet was resuspended in 10 ml ice-cold 1M sorbitol, recentrifuged, then resuspended in a final volume of 0.2 ml ice-cold 1M sorbitol. A 40 μ l aliquot of cells was placed into the chamber of a cold 0.2 cm electroporation cuvette (Invitrogen), along with 100 ng plasmid DNA for pTW113. The cuvette was placed into an
Invitrogen Electroporator II and pulsed at 1500 V, 25 μ F, 100 Ω . Electroporated cells were diluted with 0.5 ml 1M sorbitol, and 0.25 ml was spread on an SD agar plate containing 1M sorbitol. After 3 days' growth at 30°C, individual colonies were streaked on SD + CAA agar plates.

E. Induction of pTW113/ABL115, purification of KPI(-4→57)

Yeast cultures were grown in a rich broth and the galactose promoter of the KPI expression vector induced with the addition of galactose as described by Sherman, *Methods Enzymol.* 194:3 (1991). A single well-isolated colony of pTW113/ABL115 was used to inoculate a 10 ml overnight culture in Yeast Batch Medium. The next day, 1L Yeast Batch Medium which had been made 0.2% glucose was inoculated to an OD₆₀₀ of 0.1 with the overnight culture. Following 24 hours at 30°C with vigorous shaking, the 1L culture was induced by the addition of 20 ml Yeast Galactose Feed Medium. Following induction, the culture was fed every 12 hours with the addition of 20 ml Yeast Galactose Feed Medium. At 48 hours after induction, the yeast broth was harvested by centrifugation, then adjusted to pH 7.0 with 2M Tris, pH 10. The broth was subjected to trypsin-Sepharose affinity chromatography, and bound KPI(-4→57) was eluted with 20mM Tris pH 2.5. See Schilling et al., *Gene* 98:225 (1991). Final purification of KPI(-4→57) was accomplished by HPLC chromatography on a semi-prep Vydac

C4 column in a gradient of 20% to 35% acetonitrile. The sample was dried and resuspended in PBS at 1-2 mg/ml. The amino acid sequence of KPI(-4→57) is shown in Figure 8.

5 **Example 2. Recombinant Expression of site-directed KPI(-4→57) variants**

Expression vectors for the production of specific variants of KPI(-4→57) were all constructed using the pTW113 backbone as a starting point. For each KPI
10 variant, an expression construct was created by replacing the 40 bp RsrII-AatII fragment of the synthetic KPI gene contained in pTW113 with a pair of annealed oligonucleotides which encode specific codons mutated from the wild-type KPI(-4→57) sequence. In the following
15 Examples the convention used for designating the amino substituents in the KPI variants indicates first the single letter code for the amino acid found in wild-type KPI, followed by the position of the residue using the numbering convention described *supra*, followed by the
20 code for the replacement amino acid. Thus, for example, M15R indicates that the methionine residue at position 15 is replaced by an arginine.

A. Construction of pTW6165

The strategy for constructing pTW6165 is shown in
25 Figure 9. Plasmid pTW113 was digested with RsrII and AatII, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (812 + 813) was phosphorylated, annealed and gel-purified as described above.

30 812: GTCCGTGCCGTGCAGCTATCTGGCGCTGGTACTTTGACGT
813: CAAAGTACCAGCGCCAGATAGCTGCACGGCACG

The annealed oligonucleotides were ligated into the RsrII and AatII-digested pTW113, and the ligation product was used to transform *E. coli* strain MC1061. Transformed
35 colonies were selected by ampicillin resistance. The

resulting plasmid, pTW6165, encodes the 445 bp synthetic gene for the α -factor-KPI(-4 \rightarrow 57; M15A, S17W) fusion. See Figure 10.

5 B. Construction of pTW6166, pTW6175, pBG028, pTW6183, pTW6184, pTW6185, pTW6173, pTW6174.

Construction of the following KPI(-4 \rightarrow 57) variants was accomplished exactly as outlined for pTW6165. The oligonucleotides utilized for each construct are denoted below, and the sequences of annealed oligonucleotide
10 pairs are shown in Figure 11. Figures 12-19 show the synthetic genes for the α -factor fusions with each KPI(-4 \rightarrow 57) variant.

pTW6166: KPI(-4 \rightarrow 57; M15A, S17Y) — See Figure 12

814: GTCCGTGCCGTGCAGCTATCTACCGCTGGTACTTTGACGT
15 815: CAAAGTACCAGCGGTAGATAGCTGCACGGCACG

pTW6175: KPI(-4 \rightarrow 57; M15L, S17F) — See Figure 13

867: GTCCGTGCCGTGCATTGATCTTCCGCTGGTACTTTGACGT
868: CAAAGTACCAGCGGAAGATCAATGCACGGCACG

pBG028: KPI(-4 \rightarrow 57; M15L, S17Y) — See Figure 14

20 1493: GTCCGTGCCGTGCTTTGATCTACCGCTGGTACTTTGACGT
1494: CAAAGTACCAGCGGTAGATCAAAGCACGGCACG

pTW6183: KPI(-4 \rightarrow 57; I16H, S17F) — See Figure 15

925: GTCCGTGCCGTGCAATGCACTTCCGCTGGTACTTTGACGT
926: CAAAGTACCAGCGGAAGTGCAATGCACGGCACG

25 pTW6184: KPI(-4 \rightarrow 57; I16H, S17Y) — See Figure 16

927: GTCCGTGCCGTGCAATGCACTACCGCTGGTACTTTGACGT

928: CAAAGTACCAGCGGTAGTGCATTGCACGGCACG

pTW6185: KPI(-4→57; I16H, S17W) — See Figure 17

929: GTCCGTGCCGTGCAATGCACTGGCGCTGGTACTTTGACGT

930: CAAAGTACCAGCGCCAGTGCATTGCACGGCACG

5 pTW6173: KPI(-4→57; M15A, I16H) — See Figure 18

863: GTCCGTGCCGTGCAGCTCACTCCCGCTGGTACTTTGACGT

864: CAAAGTACCAGCGGGAGTGAGCTGCACGGCACG

pTW6174: KPI(-4→57; M15L, I16H) — See Figure 19

865: GTCCGTGCCGTGCATTGCACTCCCGCTGGTACTTTGACGT

10 866: CAAAGTACCAGCGGGAGTGCAATGCACGGCACG

C. Transformation of yeast with expression vectors

Yeast strain ABL115 was transformed by electroporation exactly according to the protocol described for transformation by pTW113.

15 *D. Induction of transformed yeast strains, purification of KPI(-4→57) variants.*

Cultures of yeast strains were grown and induced, and recombinant secreted KPI(-4→57) variants were purified according to the procedure described for KPI(-4→57). The amino acid sequences of KPI(-4→57) variants are shown in Figures 20-29.

Example 3. Identification of KPI (-4→57; M15A, S17F) DD185 by phage display.

A. Construction of vector pSP26:Amp:F1

25 The construction of pSP26:Amp:F1 is outlined in Figure 30. Vector pSP26:Amp:F1 contributes the basic plasmid backbone for the construction of the phage display vector for the *phoA*:KPI fusion, PDW1 #14. pSP26:Amp:F1 contains a low-copy number origin of

replication, the ampicillin-resistance gene (Amp) and the F1 origin for production of single-stranded phagemid DNA.

The ampicillin-resistance gene (Amp) was generated through polymerase chain reaction (PCR) amplification from the plasmid genome of PUC19 using oligonucleotides 176 and 177:

176: GCCATCGATGGTTTCTTAAGCGTCAGGTGGCACTTTTC
177: GCGCCAATTCTTGGTCTACGGGGTCTGACGCTCAGTGGAACGAA

The PCR amplification of Amp was done according to standard techniques, using Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). Amplification from plasmid pUC19 with these oligonucleotides yielded a fragment of 1159 bp, containing PflMI and ClaI restriction sites. The PCR product was digested with PflMI and ClaI and purified by agarose gel electrophoresis in 3% NuSieve Agarose (FMC Corp.). Bacterial expression vector pSP26 (*supra*) was digested with PflMI and ClaI and the larger vector fragment was purified. The PflMI-ClaI PCR fragment was ligated into the previously digested pSP26 containing the Amp gene. The ligation product was used to transform *E. coli* strain MC1061 and colonies were selected by ampicillin resistance. The resulting plasmid is denoted pSP26:Amp.

The F1 origin of replication from the mammalian expression vector pcDNAII (Invitrogen) was isolated in a 692 bp EarI fragment. Plasmid pcDNAII was digested with EarI and the resulting 692 bp fragment purified by agarose gel electrophoresis. EarI-NotI adapters were added to the 692 bp EarI fragment by ligation of two annealed oligonucleotide pairs, 179 + 180 and 181 + 182. The oligo pairs were annealed as described above.

179: GGCCGCTCTTCC
180: AAAGGAAGAGC
181: CTAGAATTGC
182: GGCCGCAATTC

The oligonucleotide-ligated fragment was then ligated into the single *NotI* site of *PSP26:Amp* to yield the vector *pSP26:Amp:F1*.

B. Construction of vector *pgIII*

5 The construction of *pgIII* is outlined in Figure 31. The portion of the phage *geneIII* protein gene contained by the PDW1 #14 phagemid vector was originally obtained as a PCR amplification product from vector *m13mp8*. A portion of *m13mp8 geneIII* encoding the carboxyl-terminal
10 158 amino acid residues of the *geneIII* product was isolated by PCR amplification of *m13mp8* nucleotide residues 2307-2781 using PCR oligos 6162 and 6160.

6162: GCCGGATCCGCTATTTCCGGTGGTGGCTCTGGTTCC

6160: GCCAAGCTTATTAAGACTCCTTATTACGCAG

15 The PCR oligos contain *BamHI* and *HindIII* restriction recognition sites such that PCR from *m13mp8* plasmid DNA with the oligo pair yielded a 490 bp *BamHI-HindIII* fragment encoding the appropriate portion of *geneIII*. The PCR product was ligated between the *BamHI* and *HindIII*
20 sites within the polylinker of *PUC19* to yield plasmid *pgIII*.

C. Construction of *pPhoA:KPI:gIII*

Construction of *pPhoA:KPI:gIII* is outlined in Figure 32. A portion of the *phoA* signal sequence and KPI
25 fusion encoded by the phage display vector PDW1 #14 originates with *pPhoA:KPI:gIII*. The 237 bp *NdeI-HindIII* fragment of *pTW10:KPI* encoding the entire *phoA:KPI* (1-57) fusion was isolated by preparative agarose gel electrophoresis, and inserted between the *NdeI* and
30 *HindIII* sites of *pUC19* to yield plasmid *pPhoA:KPI*. The 490 bp *BamHI-HindIII* fragment of *pgIII* encoding the C-terminal portion of the *geneIII* product was then isolated and ligated between the *BamHI* and *HindIII* sites of *pPhoA:KPI* to yield vector *pPhoA:KPI:gIII*. The
35 *pPhoA:KPI:gIII* vector encodes a 236 amino acid residue

fusion of the *phoA* signal peptide, KPI (1→57) and the carboxyl-terminal portion of the *geneIII* product.

D. Construction of pLG1

Construction of pLG1 is illustrated in Figure 33. The exact *geneIII* sequences contained in vector PDW1 #14 originate with phage display vector pLG1. A modified *geneIII* segment was generated by PCR amplification of the *geneIII* region from pgIII using PCR oligonucleotides 6308 and 6305.

6308: AGCTCCGATCTAGGATCCGGTGGTGGCTCTGGTTCCGGT
6305: GCAGCGGCCGTTAAGCTTATTAAGACTCCT

PCR amplification from pgIII with these oligonucleotides yielded a 481 bp *Bam*HI-*Hind*III fragment encoding a *geneIII* product shortened by 3 amino acid residues at the amino-terminal portion of the segment of the *geneIII* fragment encoded by pgIII. A 161 bp *Nde*I-*Bam*HI fragment was generated by PCR amplification from bacterial expression plasmid pTHW05 using oligonucleotides 6306 and 6307.

6306: GATCCTTGTGTCCATATGAAACAAAGC
6307: CACGTCGGTCGAGGATCCCTAACCACGGCCTTTAACCAG

The 161 bp *Nde*I-*Bam*HI fragment and the 481 bp *Bam*HI-*Hind*III fragment were gel-purified, and then ligated in a three-way ligation into PTW10 which had previously been digested with *Nde*I and *Hind*III. The resulting plasmid pLG1 encodes a *phoA* signal peptide-insert-*geneIII* fusion for phage display purposes.

E. Construction of pAL51

Construction of pAL51 is illustrated in Figure 34. Vector pAL51 contains the geneIII sequences of pLG1 which are to be incorporated in vector pDW1 #14.

5 A 1693 bp fragment of plasmid pBR322 was isolated, extending from the *Bam*HI site at nucleotide 375 to the *Pvu*II site at position 2064. Plasmid pLG1 was digested with *Asp*718I and *Bam*HI, removing an 87 bp fragment. The overhanging *Asp*718I end was blunted by treatment with
10 Klenow fragment, and the *Pvu*II-*Bam*HI fragment isolated from pBR322 was ligated into this vector, resulting in the insertion of a 1693 bp "stuffer" region between the *Asp*718I and *Bam*HI sites. The 78 bp *Nde*I-*Asp*718I region of the resulting plasmid was removed and replaced with
15 the annealed oligo pair 6512 + 6513.

6512: TATGAAACAAAGCACTATTGCACTGGCACTCTTACCGTTACTGTT
TACCCCGGTGACCAAAGCCCACGCTGAAG

6513: GTACCTTCAGCGTGGGCTTTGGTCACCGGGGTAAACAGTAACGGT
AAGAGTGCCAGTGCAATAGTGCTTTGTTTCA

20 The newly created 74 bp *Nde*I-*Asp*718I fragment encodes the *phoA* signal peptide, and contains a *Bst*EII cloning site. The resulting plasmid is denoted pAL51.

F. Construction of pAL53

Construction of pAL53 is outlined in Figure 35. Plasmid pAL53 contributes most of the vector sequence of
25 pDW1 #14, including the basic vector backbone with *Amp* gene, F1 origin, low copy number origin of replication, geneIII segment, *phoA* promotor and *phoA* signal sequence.

Plasmid pAL51 was digested with *Nde*I and *Hind*III and
30 the resulting 2248 bp *Nde*I-*Hind*III fragment encoding the *phoA* signal peptide, stuffer region and geneIII region was isolated by preparative agarose gel electrophoresis. The *Nde*I-*Hind*III fragment was ligated into plasmid pSP26:*Amp*:F1 between the *Nde*I and *Hind*III sites,
35 resulting in plasmid pAL52.

The *phoA* promoter region and signal peptide was generated by amplification of a portion of the *E. coli* genome by PCR, using oligonucleotide primers 405 and 406.

405: CCGGACGCGTGGAGATTATCGTCACTG
5 406: GCTTTGGTCACCGGGGTAAACAGTAACGG

The resulting PCR product is a 332 bp *MluI*-*BstEII* fragment which contains the *phoA* promoter region and signal peptide sequence. This fragment was used to replace the 148 bp *MluI*-*BstEII* segment of PAL52,
10 resulting in vector pAL53.

G. Construction of pSP26:Amp:F1:PhoA:KPI:gIII

Construction of pSP26:Amp:F1:PhoA:KPI:gIII is illustrated in Figure 36. This particular vector is the source of the KPI coding sequence found in vector pDW1
15 #14. Plasmid pPhoA:KPI:gIII was digested with *NdeI* and *HindIII*, and the resulting 714 bp *NdeI*-*HindIII* fragment was purified, and then inserted into vector pSP26:Amp:F1 between the *NdeI* and *HindIII* sites. The resulting plasmid is denoted pSP26:Amp:F1:PhoA:KPI:gIII.

20 H. Construction of pDW1 #14

Construction of pDW1 #14 is illustrated in Figure 37. The sequences encoding KPI were amplified from plasmid pSP26:Amp:F1:PhoA:KPI:gIII by PCR, using oligonucleotide primers 424 and 425.

25 424: CTGTTTACCCCGGTGACCAAAGCCGAGGTGTGCTCTGAACAA
425: AATAGCGGATCCGCACACTGCCATGCAGTACTCTTC

The resulting 172 bp *BstEII*-*BamHI* fragment encodes most of KPI (1→55). This fragment was used to replace the stuffer region in pAL53 between the *BstEII* and *BamHI*
30 sites. The resulting plasmid, PDW1 #14, is the parent KPI phage display vector for preparation of randomized KPI phage libraries. The coding region for the *phoA*-KPI (1→55)-geneIII fusion is shown in Figure 38.

I. Construction of pDW1 14-2

Construction of pDW1 14-2 is illustrated in Figure 39. The first step in the construction of the KPI phage libraries in pDW1 #14 was the replacement of the AgeI-BamHI fragment within the KPI coding sequence with a stuffer fragment. This greatly aids in preparation of randomized KPI libraries which are substantially free of contamination of phagemid genomes encoding wild-type KPI sequence.

Plasmid pDW1 #14 was digested with AgeI and BamHI, and the 135 bp AgeI-BamHI fragment encoding KPI was discarded. A stuffer fragment was created by PCR amplification of a portion of the PBR322 Tet gene, extending from the BamHI site at nucleotide 375 to nucleotide 1284, using oligo primers 266 and 252.

266: GCTTTAAACCGGTAGGTGGCCCGCTCCATGCACC

252: CGAATTCACCGGTGTCATCCTCGGCACCGTCACCCT

The resulting 894 bp AgeI-BamHI stuffer fragment was then inserted into the AgeI/BamHI-digested pDW1 #14 to yield the phagemid vector pDW1 14-2. This vector was the starting point for construction of the randomized KPI libraries.

J. Construction of KPI Library 16-19

Construction of KPI Library 16-19 is outlined in Figure 40. Library 16-19 was constructed to display KPI-geneIII fusions in which amino acid positions Ala¹⁴, Met¹⁵, Ile¹⁶ and Ser¹⁷ are randomized. For preparation of the library, plasmid pDW1 14-2 was digested with AgeI and BamHI to remove the stuffer region, and the resulting vector was purified by preparative agarose gel electrophoresis. Plasmid pDW1 #14 was used as template in a PCR amplification of the KPI region extending from the AgeI site to the BamHI site. The oligonucleotide primers used were 544 and 551.

544: GGGCTGAGACCGGTCCGTGCCGT(NNS),CGCTGGTACTTTGACGTC

551: GGAATAGCGGATCCGCACACTGCCATGCAG

Oligonucleotide primer 544 contains four randomized codons of the sequence NNS, where N represents equal mixtures of A/G/C/T and S an equal mixture of G or C. Each NNS codon thus encodes all 20 amino acids plus a single possible stop codon, in 32 different DNA sequences. PCR amplification from the wild-type KPI gene resulted in the production of a mixture of 135 bp AgeI-BamHI fragments all containing different sequences in the randomized region. The PCR product was purified by preparative agarose gel electrophoresis and ligated into the AgeI/BamHI digested PDW1 14-2 vector. The ligation mixture was used to transform *E. coli* Top10F' cells (Invitrogen) by electroporation according to the manufacturer's directions. The resulting Library 16-19 contained approximately 400,000 independent clones. The potential size of the library, based upon the degeneracy of the priming PCR oligo #544 was 1,048,576 members. The expression unit encoded by the members of Library 16-19 is shown in Figure 41.

K. Selection of Library 16-19 with human plasma kallikrein

KPI phage were prepared and amplified by infecting transformed cells with M13K07 helper phage as described by Matthews et al., *Science* 260:1113 (1993). Human plasma kallikrein (Enzyme Research Laboratories, South Bend, IN), was coupled to Sepharose 6B resin. Prior to phage binding, the immobilized kallikrein resin was washed three times with 0.5 ml assay buffer (AB = 100mM Tris-HCl, pH 7.5, 0.5M NaCl, 5mM each of KCl, CaCl₂, MgCl₂, 0.1% gelatin, and 0.05% Triton X-100). Approximately 5×10^9 phage particles of the amplified Library 16-19 in PBS, pH 7.5, containing 300mM NaCl and 0.1% gelatin, were bound to 50 μ l kallikrein resin containing 15 pmoles of active human plasma kallikrein in a total volume of 250 μ l. Phage were allowed to bind for 4 h at room temperature, with rocking. Unbound phage were removed by washing the kallikrein resin three times

in 0.5 ml AB. Bound phage were eluted sequentially by successive 5 minute washes: 0.5 ml 50mM sodium citrate, pH 6.0, 150mM NaCl; 0.5 ml 50mM sodium citrate, pH 4.0, 150mM NaCl; and 0.5 ml 50mM glycine, pH 2.0, 150mM NaCl.

5 Eluted phage were neutralized immediately and phagemids from the pH 2.0 elution were titered and amplified for reselection. After three rounds of selection on kallikrein-Sepharose, phagemid DNA was isolated from 22 individual colonies and subjected to DNA sequence
10 analysis.

The most frequently occurring randomized KPI region encoded: Ala¹⁴-Ala¹⁵-Ile¹⁶-Phe¹⁷. The *phoA*-KPI-geneIII region encoded by this class of selected KPI phage is shown in Figure 42. The KPI variant encoded by these
15 phagemids is denoted KPI (1→55; M15A, S17F).

L. Construction of pDD185 KPI (-4→57; M15A, S17F)

Figure 43 outlines the construction of pDD185 KPI (-4→57; M15A, S17F). The sequences encoding KPI (1→55; M15A, S17F) were moved from one phagemid vector, pDW1
20 (16-19) 185, to the yeast expression vector so that the KPI variant could be purified and tested.

Plasmid pTW113 encoding wild-type KPI (-4→57) was digested with AgeI and BamHI and the 135 bp AgeI-BamHI fragment was discarded. The 135 bp AgeI-BamHI fragment
25 of pDW1 (16-19) 185 was isolated and ligated into the yeast vector to yield plasmid pDD185, encoding α -factor fused to KPI (-4→57; M15A, S17F). See Figure 44.

M. Purification of KPI (-4→57; M15A, S17F) pDD185

Transformation of yeast strain ABL115 with pDD185, induction of yeast cultures, and purification of KPI
30 (-4→57; M15A, S17F) pDD185 was accomplished as described for the other KPI variants.

N. Construction of KPI Library 6 — M15A, with residues 14, 16-18 random.

35 Library 6 was constructed to display KPI-geneIII fusions in which amino acid positions Ala¹⁴, Ile¹⁶, Ser¹⁷

and Arg¹⁸ are randomized, but position 15 was held constant as Ala. For preparation of the library, plasmid pDW1 #14 was used as template in a PCR amplification of the KPI region extending from the AgeI site to the BamHI site. The oligonucleotide primers used were 551 and 1003.

1003: GCTGAGACCGGTCCGTGCCGTNNSGCA(NNS), TGGTACTTTGACGTC

551: GGAATAGCGGATCCGCACACTGCCATGCAG

Oligonucleotide primer 1003 contained four randomized codons of the sequence NNS, where N represents equal mixtures of A/G/C/T and S an equal mixture of G or C. Each NNS codon thus encodes all 20 amino acids plus a single possible stop, in 32 different DNA sequences. PCR amplification from the wild-type KPI gene resulted in the production of a mixture of 135 bp AgeI-BamHI fragments all containing different sequences in the randomized region. The PCR product was phenol extracted, ethanol precipitated, digested with BamHI and purified by preparative agarose gel electrophoresis. Plasmid pDW1 14-2 was digested with BamHI, phenol extracted and ethanol precipitated. The insert was ligated at high molar ratio to the vector which was then digested with AgeI to remove the stuffer region. The vector containing the insert was purified by agarose gel electrophoresis and recircularized. The resulting library contains approximately 5×10^6 independent clones.

O. *Construction of KPI Library 7 — residues 14-18 random.*

Library 7 was constructed to display KPI-geneIII fusions in which amino acid positions Ala¹⁴, Met¹⁵, Ile¹⁶, Ser¹⁷ and Arg¹⁸ are randomized. For preparation of the library, plasmid pDW1 #14 was used as template in a PCR amplification of the KPI region extending from the AgeI site to the BamHI site. The oligonucleotide primers used were 551 and 1179.

1179: GCTGAGACCGGTCCGTGCCGT (NNS), TGGTACTTTGACGTC

551: GGAATAGCGGATCCGCACACTGCCATGCAG

Oligonucleotide primer 1179 contains five randomized codons of the sequence NNS, where N represents equal mixtures of A/G/C/T and S an equal mixture of G or C. Each NNS codon thus encoded all 20 amino acids plus a single possible stop, in 32 different DNA sequences. PCR amplification from the wild-type KPI gene resulted in the production of a mixture of 135 bp AgeI-BamHI fragments all containing different sequences in the randomized region. The PCR product was phenol extracted, ethanol precipitated, digested with BamHI and purified by preparative agarose gel electrophoresis. Plasmid pDW1 14-2 was digested with BamHI, phenol extracted and ethanol precipitated. The insert was ligated at high molar ratio to the vector which was then digested with AgeI to remove the stuffer region. The vector containing the insert was purified by agarose gel electrophoresis and recircularized. The resulting library contains approximately 1×10^7 independent clones.

P. Selection of Libraries 6 & 7 with human factor XIIa

KPI phage were prepared and amplified by infecting transformed cells with M13K07 helper phage (Matthews and Wells, 1993). Human factor XIIa (Enzyme Research Laboratories, South Bend, IN), was biotinylated as follows. Factor XIIa (0.5 mg) in 5mM sodium acetate pH 8.3 was incubated with Biotin Ester (Zymed) at room temperature for 1.5 h, then buffer-exchanged into assay buffer (AB). Approximately 1×10^{10} phage particles of each amplified Library 6 or 7 in PBS, pH 7.5, containing 300mM NaCl and 0.1% gelatin, were incubated with 50 pmoles of active biotinylated human factor XIIa in a total volume of 200 μ l. Phage were allowed to bind for 2 h at room temperature, with rocking. Following the binding period, 100 μ l Strepavidin Magnetic Particles (Boehringer

Mannheim) were added to the mixture and incubated at room temperature for 30 minutes. Separation of magnetic particles from the supernatant and wash/elution buffers was carried out using MPC-E-1 Neodymium-iron-boron permanent magnets (Dynal). Unbound phage were removed by washing the magnetically bound biotinylated XIIa-phage complexes three times with 0.5 ml AB. Bound phage were eluted sequentially by successive 5 minute washes: 0.5 ml 50mM sodium citrate, pH 6.0, 150mM NaCl; 0.5 ml 50mM sodium citrate, pH 4.0, 150mM NaCl; and 0.5 ml 50mM glycine, pH 2.0, 150mM NaCl. Eluted phage were neutralized immediately and phagemids from the pH 2.0 elution were titered and amplified for reselection. After 3 or 4 rounds of selection with factor XIIa, phagemid DNA was isolated from individual colonies and subjected to DNA sequence analysis.

Sequences in the randomized regions were compared with one another to identify consensus sequences appearing more than once. From Library 6 a phagemid was identified which encoded M15L, S17Y, R18H. From Library 7 a phagemid was identified which encoded M15A, S17Y, R18H.

Q. Construction of pBG015 KPI (-4→57; M15L, S17Y, R18H), pBG022 (-4→57; M15A, S17Y, R18H)

The sequences encoding KPI (1→55; M15L, S17Y, R18H) and KPI (1→55; M17A, S17Y, R18H) were moved from the phagemid vectors to the yeast expression vector so that the KPI variant could be purified and tested.

Plasmid pTW113 encoding wild-type KPI (-4→57) was digested with AgeI and BamHI and the 135 bp AgeI-BamHI fragment was discarded. The 135 bp AgeI-BamHI fragment of the phagemid vectors were isolated and ligated into the yeast vector to yield plasmids pBG015 and pBG022, encoding alpha-factor fused to KPI (-4→57; M15L, S17Y, R18H), and KPI (-4→57; M15A, S17Y, R18H), respectively.

R. Construction of pBG029 KPI (-4→57, T9V, M15L, S17Y, R18H)

Plasmid pBG015 was digested with *Xba*I and *Rsr*II, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (1593 + 1642) was phosphorylated, annealed and gel-purified as described previously.

1593: CTAGATAAAAGAGAGGTTGTTAGAGAGGTGTGCTCTGAACAAGCT
GAGGTTG

1642: GACCAACCTCAGCTTGTTTCAGAGCACACCTCTCTAA
CAACCTCTCTTTTAT

The annealed oligonucleotides were ligated into the *Xba*I and *Rsr*II-digested pBG015, and the ligation product was used to transform *E. coli* strain MC1061 to ampicillin resistance. The resulting plasmid pBG029, encodes the 445 bp synthetic gene for the alpha-factor-KPI (-4→57; T9V, M15L, S17F, R18H) fusion.

S. Construction of pBG033 KPI (-4→57; T9V, M15A, S17Y, R18H)

Plasmid pBG022 was digested with *Xba*I and *Rsr*II, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (1593 + 1642) was phosphorylated, annealed and gel-purified as described previously. The annealed oligonucleotides were ligated into the *Xba*I and *Rsr*II-digested pBG022, and the ligation product was used to transform *E. coli* strain MC1061 to ampicillin resistance. The resulting plasmid pBG033, encodes the 445 bp synthetic gene for the alpha-factor-KPI (-4→57; T9V, M15A, S17F, R18H) fusion.

T. Selection of Library 16-19 with human factor Xa

KPI phage were prepared and amplified by infecting transformed cells with M13K07 helper phage (Matthews and Wells, 1993). Human factor Xa (Haematologic Technologies, Inc., Essex Junction, VT) was coupled to Sepharose 6B resin. Prior to phage binding, the

immobilized Xa resin was washed three times with 0.5 ml assay buffer (AB = 100mM Tris-HCl, pH 7.5, 0.5M NaCl, 5mM each of KCl, CaCl₂, MgCl₂, 0.1% gelatin, and 0.05% Triton X-100). Approximately 4×10^{10} phage particles of the amplified Library 16-19 in PBS, pH 7.5, containing 300mM NaCl and 0.1% gelatin, were bound to 50 μ l Xa resin in a total volume of 250 μ l. Phage were allowed to bind for 4 h at room temperature, with rocking. Unbound phage were removed by washing the Xa resin three times in 0.5 ml AB. Bound phage were eluted sequentially by successive 5 minute washes: 0.5 ml 50mM sodium citrate, pH 6.0, 150mM NaCl; 0.5 ml 50mM sodium citrate, pH 4.0 150mM NaCl; and 0.5 ml 50mM glycine, pH 2.0, 150mM NaCl. Eluted phage were neutralized immediately and phagemids from the pH 2.0 elution were titered and amplified for reselection. After three rounds of selection on Xa-Sepharose, phagemid DNA was isolated and subjected to DNA sequence analysis.

Sequences in the randomized Ala¹⁴-Ser¹⁷ region were compared with one another to identify consensus sequences appearing more than once. A phagemid was identified which encoded KPI (1→55; M15L, I16F, S17K).

U. Construction of pDD131 KPI (-4→57; M15L, I16F, S17K)

The sequences encoding KPI (1→55; M15L, I16F, S17K) were moved from the phagemid vector to the yeast expression vector so that the KPI variant could be purified and tested.

Plasmid pTW113 encoding wild-type KPI (-4→57) was digested with AgeI and BamHI and the 135 bp AgeI-BamHI fragment was discarded. The 135 bp AgeI-BamHI fragment of the phagemid vector was isolated and ligated into the yeast vector to yield plasmid pDD131, encoding alpha-factor fused to KPI (-4→57; M15L, I16F, S17K).

V. Construction of pDD134 KPI (-4→57; M15L, I16F, S17K, G37Y)

Plasmid pDD131 was digested with AatI and BamHI, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (738 + 739) was phosphorylated, annealed and gel-purified as described previously.

738: CACTGAAGGTAAGTGCCTCCATTCTTTACGGCGGTTGCTACGGCAACCGT
AACAACTTTGACACTGAAGAGTACTGCATGGCAGTGTGCG

739: GATCCGCACACTGCCATGCAGTACTCTTCAGTGTCAAAGTTGTTACGGTTGC
CGTAGCAACCGCCGTAAAAGAATGGAGCGCACTTACCTTCAGTGACGT

The annealed oligonucleotides were ligated into the AatI and BamHI-digested pDD131, and the ligation product was used to transform *E. coli* strain MC1061 to ampicillin resistance. The resulting plasmid pDD134, encodes the 445 bp synthetic gene for the alpha-factor-KPI (-4→57; M15L, I16F, S17K, G37Y) fusion.

W. Construction of pDD135 KPI (-4→57; M15L, I16F, S17K, G37L)

Plasmid pDD131 was digested with AatII and BamHI, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (724 + 725) was phosphorylated, annealed and gel-purified as described previously.

738: CACTGAAGGTAAGTGCCTCCATTCTTTACGGCGGTTGCTACGGCAACCGT
AACAACTTTGACACTGAAGAGTACTGCATGGCAGTGTGCG

739: GATCCGCACACTGCCATGCAGTACTCTTCAGTGTCAAAGTTGTTACGGTTGC
CGTAGCAACCGCCGTAAAAGAATGGAGCGCACTTACCTTCAGTGACGT

The annealed oligonucleotides were ligated into the AatII and BamHI-digested pDD131, and the ligation product was used to transform *E. coli* strain MC1061 to ampicillin resistance. The resulting plasmid pDD135, encodes the

445 bp synthetic gene for the alpha-factor-KPI (-4→57; M15L, I16F, S17K, G37L) fusion.

Example 4. Kinetic analysis of KPI(-4→57) variants

The concentrations of active human plasma kallikrein, factor XIIa, and trypsin were determined by titration with p-nitrophenyl p'-guanidinobenzoate as described by Bender et al., *supra*, and Chase et al., *Biochem. Biophys. Res. Commun.* 29:508 (1967). Accurate concentrations of active KPI(-4→57) inhibitors were determined by titration of the activity of a known amount of active-site-titrated trypsin. For testing against kallikrein and trypsin, each KPI(-4→57) variant (0.5 to 100nM) was incubated with protease in low-binding 96-well microtiter plates at 30°C for 15-25 min, in 100mM Tris-HCl, pH 7.5, with 500mM NaCl, 5mM KCl, 5mM CaCl₂, 5mM MgCl₂, 0.1% Difco gelatin, and 0.05% Triton X-100. Chromogenic synthetic substrate was then be added, and initial rates at 30°C recorded by the SOFTmax kinetics program via a THERMOMax microplate reader (Molecular Devices Corp., Menlo Park, CA). The substrates used were N-α-benzoyl-L-Arg p-nitroanilide (1mM) for trypsin (20nM), and N-benzoyl-Pro-Phe-Arg p-nitroanilide (0.3mM) for plasma kallikrein (1nM). The Enzfitter (Elsevier) program was used both to plot fractional activity (i.e., activity with inhibitor, divided by activity without inhibitor), a, versus total concentration of inhibitor, I_t, and to calculate the dissociation constant of the inhibitor (K_i) by fitting the curve to the following equation:

$$a = 1 - \frac{[E]_t + [I]_t + K_i - \sqrt{([E]_t + [I]_t + K_i)^2 - 4[E]_t[I]_t}}{2[E]_t}$$

The K_is determined for purified KPI variants are shown in Figure 45. The most potent variants, KPI (-4→57; M15A, S17F) DD185 and KPI (-4→57; M15A, S17Y) TW6166 are 115-fold and 100-fold more potent,

respectively, as a human kallikrein inhibitor than wild-type KPI (-4→57). The least potent variant, KPI (-4→57; I16H, S17W) TW6185 is still 35-fold more potent than wild-type KPI.

5 For testing against factor XIIa, essentially the same reaction conditions were used, except that the substrate was N-benzoyl-Ile-Glu-Gly-Arg p-nitroaniline hydrochloride and its methyl ester (obtained from Pharmacia Hepar, Franklin, OH), and corn trypsin
10 inhibitor (Enzyme Research Laboratories, South Bend, IN) was used as a control inhibitor. Factor XIIa was also obtained from Enzyme Research Laboratories.

Various data for inhibition of the serine proteases of interest kallikrein, plasmin, and factors Xa, XIa, and
15 XIIa by a series of KPI variants are given in Figure 46. The results indicate that KPI variants can be produced that can bind to and preferably inhibit the activity of serine proteases. The results also indicate that the peptides of the invention may exhibit the preferable more
20 potent and specific inhibition of one or more serine proteases of interest.

Example 5. Effect of KPI variant KPI185-1 on postoperative bleeding

A randomized, double-blinded study using an acute
25 porcine cardiopulmonary bypass (CPB) model was used to investigate the effect of KPI185-1 on postoperative bleeding. Sixteen pigs (55-65 kg) underwent 60 minutes of hypothermic (28°C) open-chest CPB with 30 minutes of cardioplegic cardiac arrest. Pigs were randomized
30 against a control solution of physiological saline (NS; n=8) or KPI-185 (n=8) groups. During aortic cross-clamping, the tricuspid valve was inspected through an atriotomy which was subsequently repaired. Following reversal of heparin with protamine, dilateral
35 thoracostomy tubes were placed and shed blood collected for 3 hours. Shed blood volume and hemoglobin (Hgb) loss were calculated from total chest tube output and residual intrathoracic blood at time of sacrifice.

Total blood loss was significantly reduced in the KPI185-1 group (245.75 ± 66.24 ml vs. 344.25 ± 63.97 ml, $p=0.009$). In addition, there was a marked reduction in total Hgb loss in the treatment group (13.59 ± 4.26 gm vs. 23.61 ± 4.69 gm, $p=0.0005$). Thoracostomy drainage Hgb was significantly increased at 30 and 60 minutes in the control group [6.89 ± 1.44 vs. 4.41 ± 1.45 gm/dl ($p=0.004$) and 7.6 ± 1.03 vs. 5.26 ± 1.04 gm/dl ($p=0.0002$), respectively]. Preoperative and post-CPB hematocrits were not statistically different between the groups. These results are shown in graphical form in Figures 47-50.

The invention has been disclosed broadly and illustrated in reference to representative embodiments described above. Those skilled in the art will recognize that various modifications can be made to the present invention without departing from the spirit and scope thereof.

What Is Claimed Is:

1. A protease inhibitor comprising the sequence:

X¹-Val-Cys-Ser-Glu-Gln-Ala-Glu-X²-Gly-X³-
Cys-Arg-Ala-X⁴-X⁵-X⁶-X⁷-Trp-Tyr-Phe-Asp-
Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-X⁸-
Tyr-Gly-Gly-Cys-X⁹-X¹⁰-X¹¹-X¹²-Asn-Asn-Phe-
Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-
Gly-Ser-Ala-Ile,

wherein:

X¹ is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu;

X² is selected from Thr, Val, Ile and Ser;

X³ is selected from Pro and Ala;

X⁴ is selected from Arg, Ala, Leu, Gly, or Met;

X⁵ is selected from Ile, His, Leu, Lys, Ala, or Phe;

X⁶ is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu;

X⁷ is selected from Arg, His, or Ala;

X⁸ is selected from Phe, Val, Leu, or Gly;

X⁹ is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr;

X¹⁰ is selected from Ala, Arg, or Gly;

X¹¹ is selected from Lys, Ala, or Asn;

X¹² is selected from Ser, Ala, or Arg;

provided that:

when X⁴ is Arg, X⁶ is Ile;

when X⁹ is Arg, X⁴ is Ala or Leu; when X⁹ is Tyr, X⁴ is Ala or X⁵ is His; and

either X⁵ is not Ile; or X⁶ is not Ser; or X⁹ is not Leu, Phe, Met, Tyr, or Asn; or X¹⁰ is not Gly; or X¹¹ is not Asn; or X¹² is not Arg.

2. A protease inhibitor comprising the sequence:

X¹-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-
Pro-Cys-Arg-Ala-X²-X³-X⁴-Arg-Trp-Tyr-Phe-
Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-
Phe-Tyr-Gly-Gly-Cys-X⁵-Gly-Asn-Arg-Asn-

Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-
Val-Cys-Gly-Ser-Ala-Ile,

wherein:

X¹ is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu;

X² is selected from Ala, Leu, Gly, or Met;

X³ is selected from Ile, His, Leu, Lys, Ala, or Phe;

X⁴ is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu;

X⁵ is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr;

provided that:

when X⁵ is Arg, X² is Ala or Leu; when X⁵ is Tyr, X² is Ala or X³ is His; and

either X³ is not Ile; or X⁴ is not Ser; or X⁵ is not Leu, Phe, Met, Tyr, or Asn.

3. A protease inhibitor comprising the sequence:

Glu-Val-Val-Arg-Glu-Val-Cys-Ser-Glu-Gln-
Ala-Glu-Thr-Gly-Pro-Cys-Arg-Ala-X¹-X²-X³-
Arg-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-
Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-X⁴-
Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-
Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein:

X¹ is selected from Ala, Leu, Gly, or Met;

X² is selected from Ile, His, Leu, Lys, Ala, or Phe;

X³ is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu;

X⁴ is selected from Gly, Arg, Leu, Met, or Tyr;

provided that:

when X¹ is Ala, X² is Ile, His, or Leu;

when X¹ is Leu, X² is Ile or His;

when X¹ is Leu and X² is Ile, X³ is not Ser;

when X¹ is Gly, X² is Ile;

when X⁴ is Arg, X¹ is Ala or Leu;

when X⁴ is Tyr, X¹ is Ala or X² is His; and

either X¹ is not Met, or X² is not Ile, or X³ is not Ser, or X⁴ is not Gly.

4. A protease inhibitor according to claim 1, wherein at least two amino acid residues selected from the group consisting of X⁴, X⁵, X⁶, and X⁷ differ from the residues found in the naturally occurring sequence of KPI.

5. A protease inhibitor according to claim 1, wherein X¹ is Asp or Glu, X² is Thr, X³ is Pro, and X¹² is Ser.

6. A protease inhibitor according to claim 5, wherein X¹ is Glu, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, and X¹¹ is Asn.

7. A protease inhibitor according to claim 5, wherein X¹ is Asp, X² is Thr, X³ is Pro, X⁴ is Arg, X⁵ is Ile, X⁶ is Ile, X⁷ is Arg, X⁸ is Val, X⁹ is Arg, X¹⁰ is Ala, and X¹¹ is Lys.

8. A protease inhibitor according to claim 1, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Asn, and X¹² is Ala.

9. A protease inhibitor according to claim 1, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Ala, and X¹² is Arg.

10. A protease inhibitor according to claim 1, wherein X¹ is Glu, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Ala, X¹¹ is Asn, and X¹² is Arg.

11. A protease inhibitor according to claim 1, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Arg, X¹¹ is Asn, and X¹² is Arg.

12. A protease inhibitor according to claim 1, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Val, Leu, or Gly, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Asn, and X¹² is Arg.

13. A protease inhibitor according to claim 1, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Ala, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Asn, and X¹² is Arg.

14. A protease inhibitor according to claim 1, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, Val, or Ser, X³ is Pro, X⁴ is Ala or Leu, X⁵ is Ile, X⁶ is Tyr, X⁷ His, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Ala, and X¹² is Arg.

15. A protease inhibitor according to claim 14, wherein X² is Thr, and X⁴ is Ala.

16. A protease inhibitor according to claim 14, wherein X² is Thr, and X⁴ is Leu.

17. A protease inhibitor according to claim 14, wherein X² is Val, and X⁴ is Ala.

18. A protease inhibitor according to claim 14, wherein X² is Ser, and X⁴ is Ala.

19. A protease inhibitor according to claim 14, wherein X² is Val, and X⁴ is Leu.

20. A protease inhibitor according to claim 14, wherein X² is Ser, and X⁴ is Leu.

21. A protease inhibitor according to claim 1, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Leu, X⁵ is Phe, X⁶ is Lys, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Ala, and X¹² is Arg.

22. A protease inhibitor according to claim 1, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Leu, X⁵ is Phe, X⁶ is Lys, X⁷ is Arg, X⁸ is Phe, X⁹ is Tyr, X¹⁰ is Gly, X¹¹ is Ala, and X¹² is Arg.

23. A protease inhibitor according to claim 1, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Leu, X⁵ is Phe, X⁶ is Lys, X⁷ is Arg, X⁸ is Phe, X⁹ is Leu, X¹⁰ is Gly, X¹¹ is Ala, and X¹² is Arg.

24. A protease inhibitor according to claim 2, wherein X¹ is Glu, X² is Met, X³ is Ile, X⁴ is Ile, and X⁵ is Gly.

25. A protease inhibitor according to claim 3, wherein X¹ is Met, X³ is Ser, and X⁴ is Gly.

26. A protease inhibitor according to claim 25, wherein X² is selected from His, Ala, Phe, Lys, and Leu.

27. A protease inhibitor according to claim 26, wherein X² is His.

28. A protease inhibitor according to claim 27, wherein X² is Ala.

29. A protease inhibitor according to claim 27, wherein X² is Phe.

30. A protease inhibitor according to claim 27, wherein X² is Lys.

31. A protease inhibitor according to claim 27, wherein X² is Leu.

32. A protease inhibitor according to claim 3, wherein X¹ is Met, X² is Ile, and X⁴ is Gly.

33. A protease inhibitor according to claim 32, wherein X³ is Ile.

34. A protease inhibitor according to claim 32, wherein X³ is Pro.

35. A protease inhibitor according to claim 32, wherein X³ is Phe.

36. A protease inhibitor according to claim 32, wherein X³ is Tyr.

37. A protease inhibitor according to claim 32, wherein X³ is Trp.

38. A protease inhibitor according to claim 32, wherein X³ is Asn.

39. A protease inhibitor according to claim 32, wherein X³ is Leu.

40. A protease inhibitor according to claim 32, wherein X³ is Lys.

41. A protease inhibitor according to claim 32, wherein X³ is His.

42. A protease inhibitor according to claim 32, wherein X³ is Glu.

43. A protease inhibitor according to claim 3, wherein X¹ is Ala.

44. A protease inhibitor according to claim 43, wherein X² is Ile.

45. A protease inhibitor according to claim 44, wherein X³ is Phe, and X⁴ is Gly.

46. A protease inhibitor according to claim 44, wherein X³ is Tyr, and X⁴ is Gly.

47. A protease inhibitor according to claim 44, wherein X³ is Trp, and X⁴ is Gly.

48. A protease inhibitor according to claim 44, wherein X³ is Ser or Phe, and X⁴ is Arg or Tyr.

49. A protease inhibitor according to claim 43, wherein X² is His or Leu, X³ is Phe, and X⁴ is Gly.

50. A protease inhibitor according to claim 3, wherein X¹ is Leu.

51. A protease inhibitor according to claim 50, wherein X² is His, X³ is Asn or Phe, and X⁴ is Gly.

52. A protease inhibitor according to claim 50, wherein X² is Ile, X³ is Pro, and X⁴ is Gly.

53. A protease inhibitor according to claim 3, wherein X¹ is Gly, X² is Ile, X³ is Tyr, and X⁴ is Gly.

54. A protease inhibitor according to claim 3, wherein X¹ is Met, X² is His, X³ is Ser, and X⁴ is Tyr.

55. An isolated DNA molecule comprising a DNA sequence encoding a protease inhibitor according to claim 1.

56. An isolated DNA molecule according to claim 55, operably linked to a regulatory sequence that controls expression of the coding sequence in a host cell.

57. An isolated DNA molecule according to claim 56, further comprising a DNA sequence encoding a secretory signal peptide.

58. An isolated DNA molecule according to claim 57, wherein said secretory signal peptide comprises the signal sequence of yeast alpha-mating factor.

59. A host cell transformed with a DNA molecule according to claim 55.

60. A host cell according to claim 59, wherein said host cell is *E. coli* or a yeast cell.

61. A host cell according to claim 60, wherein said yeast cell is selected from *Pichia pastoris* and *Saccharomyces cerevisiae*.

62. A method for producing a protease inhibitor, comprising the steps of culturing a host cell according to claim 59 and isolating and purifying said protease inhibitor.

63. A pharmaceutical composition, comprising a protease inhibitor according to claim 1, together with a pharmaceutically acceptable sterile vehicle.

64. A method of treatment of a clinical condition associated with increased activity of one or more serine proteases, comprising administering to a patient suffering from said clinical condition an effective amount of a pharmaceutical composition according to claim 63.

65. The method of treatment of claim 64, wherein said clinical condition is blood loss during surgery.

66. A method for inhibiting the activity of serine proteases of interest in a mammal comprising administering a therapeutically effective dose of a pharmaceutical composition according to claim 63.

67. The method of claim 66, wherein said serine proteases are selected from the group consisting of: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

68. A protease inhibitor comprising the sequence:

X¹-Val-Cys-Ser-Glu-Gln-Ala-Glu-X²-Gly-Pro-
Cys-Arg-Ala-X³-X⁴-X⁵-X⁶-Trp-Tyr-Phe-Asp-
Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-
Tyr-Gly-Gly-Cys-X⁷-Gly-Asn-Arg-Asn-Asn-
Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-
Cys-Gly-Ser-Ala-Ile,

wherein:

X¹ is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu;

X² is selected from Thr, Val, Ile and Ser;

X³ is selected from Arg, Ala, Leu, Gly, or Met;

X⁴ is selected from Ile, His, Leu, Lys, Ala, or Phe;

X⁵ is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu;

X⁶ is selected from Arg, His, or Ala; and

X⁷ is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr.

69. A protease inhibitor according to claim 68, wherein at least two amino acid residues selected from the group consisting of X³, X⁴, X⁵, and X⁶ differ from the residues found in the naturally occurring sequence of KPI.

70. A protease inhibitor according to claim 68, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, Val, or Ser, X³ is Ala or Leu, X⁴ is Ile, X⁵ is Tyr, X⁶ is His and X⁷ is Gly.

71. A protease inhibitor according to claim 70, wherein X² is Thr, and X³ is Ala.

72. A protease inhibitor according to claim 70, wherein X² is Thr, and X³ is Leu.

73. A protease inhibitor according to claim 70, wherein X² is Val, and X³ is Ala.

74. A protease inhibitor according to claim 70, wherein X² is Ser, and X³ is Ala.

75. A protease inhibitor according to claim 70, wherein X² is Val, and X³ is Leu.

76. A protease inhibitor according to claim 70, wherein X² is Ser, and X³ is Leu.

77. A protease inhibitor according to claim 68, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Leu, X⁴ is Phe, X⁵ is Lys, X⁶ is Arg and X⁷ is Gly.

78. A protease inhibitor according to claim 68, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Leu, X⁴ is Phe, X⁵ is Lys, X⁶ is Arg and X⁷ is Tyr.

79. A protease inhibitor according to claim 68, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Leu, X⁴ is Phe, X⁵ is Lys, X⁶ is Arg and X⁷ is Leu.

80. A protease inhibitor comprising the sequence:

X¹-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-
Pro-Cys-X²-Ala-X³-X⁴-X⁵-X⁶-Trp-Tyr-Phe-Asp-
Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-
Tyr-Gly-Gly-Cys-Gly-Gly-Asn-Arg-Asn-Asn-
Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-
Cys-Gly-Ser-Ala-Ile,

wherein:

X¹ is selected from Glu-Val-Val-Arg-Glu- and Asp-Val-Val-Arg-Glu-;

X² is selected from Arg and Lys;

X³ is selected from Met, Arg, Ala, Leu, Ser, Val;

X⁴ is selected from Ile and Ala;

X⁵ is selected from Ser, Ile, Ala, Pro, Phe, Tyr, and Trp; and

X⁶ is selected from Arg, Ala, His, Gln, and Thr; provided that:

when X² is Arg, X³ is Leu, and X⁴ is Ile, X⁵ cannot be Ser; and also provided that either X³ is not Met; or X⁴ is not Ile; or X⁵ is not Ser; or X⁶ is not Arg.

81. A protease inhibitor according to claim 80, wherein X⁵ is selected from Phe, Tyr and Trp.

82. A protease inhibitor according to claim 80, wherein X⁴ is Ile.

83. A protease inhibitor according to claim 82, wherein X² is Lys.

84. A protease inhibitor according to claim 83, wherein X³ is Met.

85. A protease inhibitor according to claim 84, wherein X⁵ is Ser.

86. A protease inhibitor according to claim 84, wherein X⁵ is Ile.

87. A protease inhibitor according to claim 83, wherein X³ is Arg.

88. A protease inhibitor according to claim 87, wherein X⁵ is Ser.

89. A protease inhibitor according to claim 87, wherein X⁵ is Ile.

90. A protease inhibitor according to claim 82, wherein X² is Arg.

91. A protease inhibitor according to claim 90, wherein X³ is Arg or Met, and X⁵ is Ser or Ile.

92. A protease inhibitor according to claim 91, wherein X³ is Arg.

93. A protease inhibitor according to claim 92, wherein X⁵ is Ser.

94. A protease inhibitor according to claim 92, wherein X⁵ is Ile.

95. A protease inhibitor according to claim 91, wherein X³ is Met.

96. A protease inhibitor according to claim 95, wherein X⁵ is Ser.

97. A protease inhibitor according to claim 95, wherein X⁵ is Ile.

98. A protease inhibitor according to claim 82, wherein X³ is Ala.

99. A protease inhibitor according to claim 82, wherein X³ is Leu.

100. A protease inhibitor according to claim 82, wherein X³ is Ser.

101. A protease inhibitor according to claim 82, wherein X³ is Val.

102. A protease inhibitor according to claim 82, wherein X³ is Pro.

103. A protease inhibitor according to claim 82, wherein X⁵ is Phe.

104. A protease inhibitor according to claim 82, wherein X⁵ is Tyr.

105. A protease inhibitor according to claim 82, wherein X⁵ is Trp.

106. A protease inhibitor according to claim 104, wherein X³ is Ala or Leu.

107. A protease inhibitor according to claim 106, wherein X³ is Ala.

108. A protease inhibitor according to claim 106, wherein X³ is Leu.

109. A protease inhibitor according to claim 105, wherein X³ is Ala.

110. A protease inhibitor according to claim 109, wherein X⁵ is His.

111. A protease inhibitor according to claim 109, wherein X⁵ is Gln.

112. A protease inhibitor according to claim 109, wherein X⁵ is Thr.

113. An isolated DNA molecule comprising a DNA sequence encoding a protease inhibitor according to claim 80.

114. An isolated DNA molecule according to claim 113, operably linked to a regulatory sequence that controls expression of the coding sequence in a host cell.

115. An isolated DNA molecule according to claim 114, further comprising a DNA sequence encoding a secretory signal peptide.

116. An isolated DNA molecule according to claim 115, wherein said secretory signal peptide comprises the signal sequence of yeast alpha-mating factor.

117. A host cell transformed with a DNA molecule according to claim 113.

118. A host cell according to claim 117, wherein said host cell is *E. coli* or a yeast cell.

119. A host cell according to claim 118, wherein said yeast cell is selected from *Pichia pastoris* and *Saccharomyces cerevisiae*.

120. A method for producing a protease inhibitor, comprising the steps of culturing a host cell according to claim 117 and isolating and purifying said protease inhibitor.

121. A pharmaceutical composition, comprising a protease inhibitor according to claim 80, together with a pharmaceutically acceptable sterile vehicle.

122. A method of treatment of a clinical condition associated with increased activity of one or more serine proteases, comprising administering to a patient suffering from said clinical condition an effective amount of a pharmaceutical composition according to claim 121.

123. The method of treatment of claim 122, wherein said clinical condition is blood loss during surgery.

124. A method for inhibiting the activity of serine proteases of interest in a mammal comprising

administering a therapeutically effective dose of a pharmaceutical composition according to claim 121.

125. The method of claim 124, wherein said serine proteases are selected from the group consisting of: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

126. A protease inhibitor according to claim 81, wherein X⁴ is Ile.

127. A protease inhibitor according to claim 126, wherein X⁵ is Phe.

128. A protease inhibitor according to claim 126, wherein X⁵ is Tyr.

129. A protease inhibitor according to claim 126, wherein X⁵ is Trp.

130. A protease inhibitor according to claim 128, wherein X³ is Ala or Leu.

131. A protease inhibitor according to claim 130, wherein X³ is Ala.

132. A protease inhibitor according to claim 130, wherein X³ is Leu.

133. A protease inhibitor according to claim 129, wherein X³ is Ala.

1/59

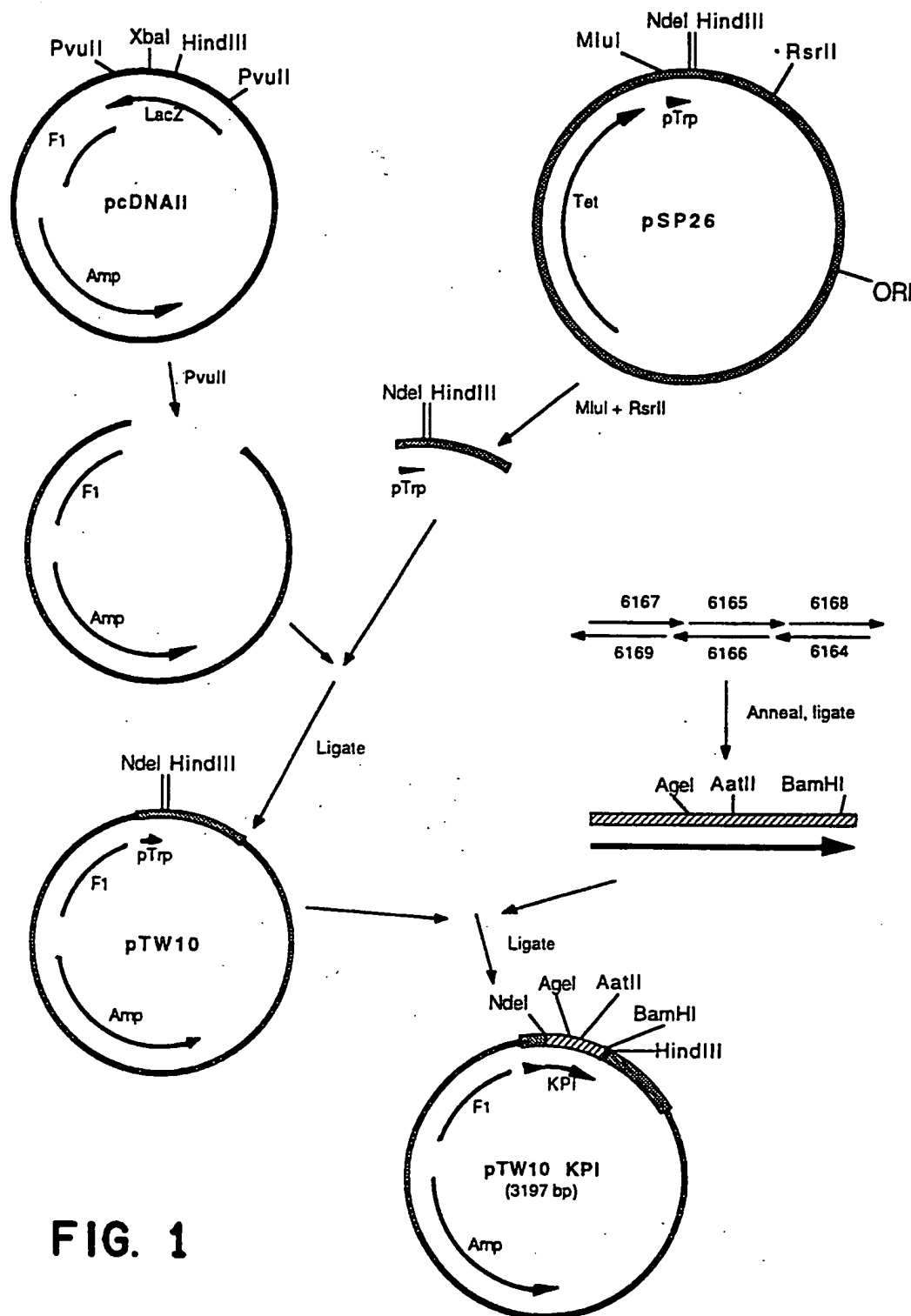


FIG. 1

2 / 59

FIG. 2

NdeI
TATG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAA
AC TTT GTT TCG TGA TAA CGT GAC GAG AAT GGC AAT GAC AAA TGG GGA CAC TGT TTT
▶ Met Lys Gln Ser Thr Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys

KPI
GAG GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT GCA ATG ATC TCC CGC TGG
CGC CTC CAC ACG AGA CTT GTT CGA CTC TGG CCA GGC ACG GCA CGT TAC TAG AGG GCG ACC
▶ Ala Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met Ile Ser Arg Trp

AgeI
GAG GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT GCA ATG ATC TCC CGC TGG
CGC CTC CAC ACG AGA CTT GTT CGA CTC TGG CCA GGC ACG GCA CGT TAC TAG AGG GCG ACC
▶ Ala Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met Ile Ser Arg Trp

AatII
TAC TTT GAC CTC ACT GAA GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC AAC
ATG AAA CTG CAG TGA CTT CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG TTG
▶ Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Asn

BamHI
CGT AAC AAC TTT GAC ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TA
GCA TTG TTG AAA CTG TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA
▶ Arg Asn Asn Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

HindIII
CGT AAC AAC TTT GAC ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TA
GCA TTG TTG AAA CTG TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA
▶ Arg Asn Asn Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

3/59

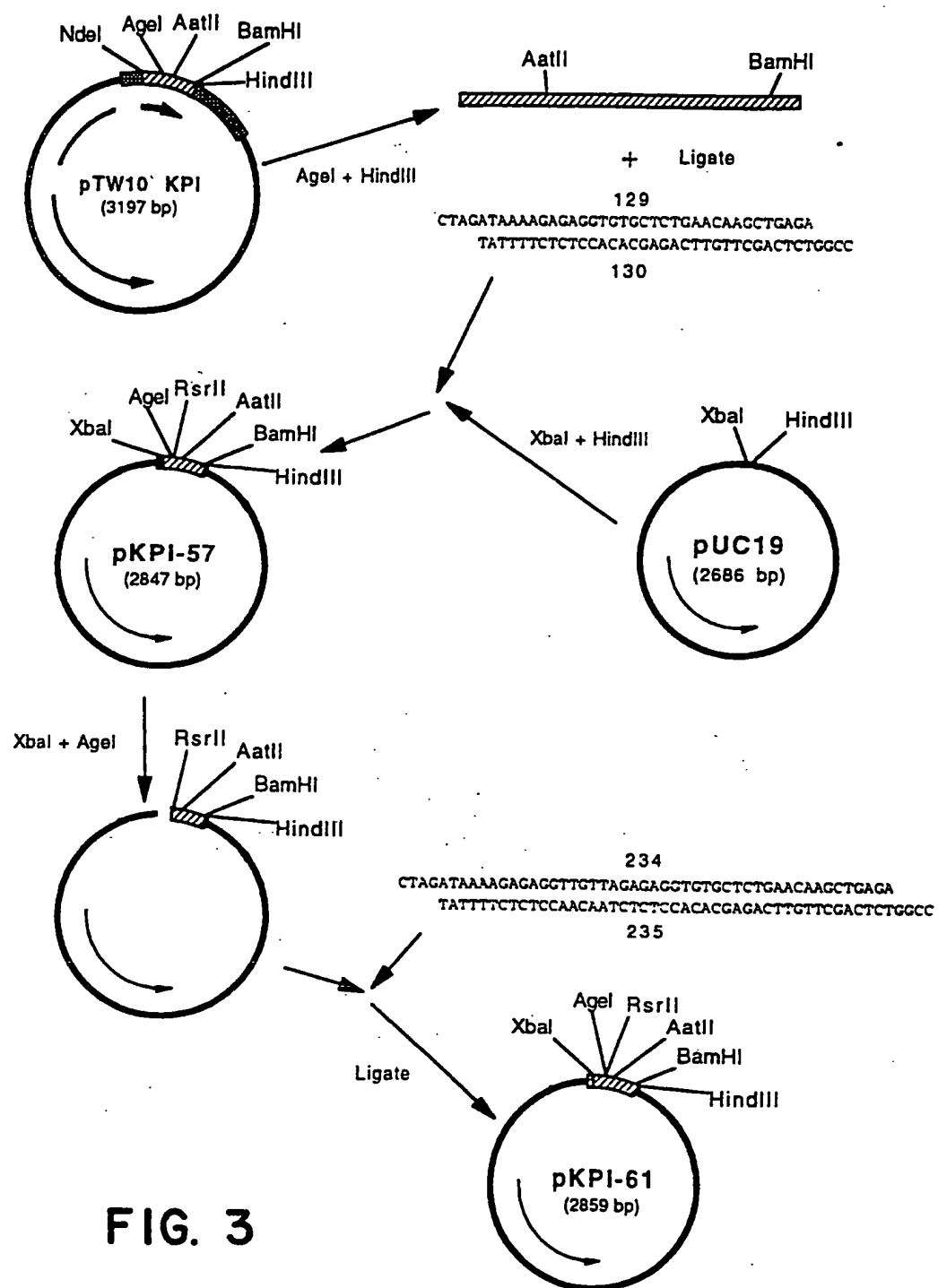


FIG. 3

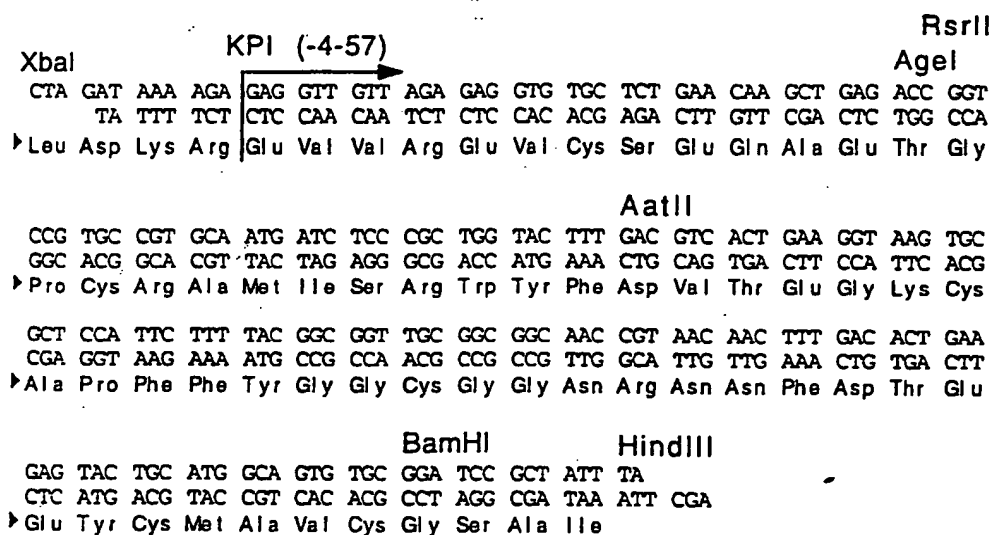
4/59

FIG. 4

| XbaI | | | | KPI (1-57) | | | | RsrII | | | | | | | | | AgeI | | | |
|-------|-----|-----|-----|------------|-----|-----|-----|---------|-----|-----|-----|-----|-----|-----|-----|-----|------|--|--|--|
| CTA | GAT | AAA | AGA | GAG | GTG | TGC | TCT | GAA | CAA | GCT | GAG | ACC | GGT | CCG | TGC | CGT | | | | |
| | TA | TTT | TCT | CTC | CAC | ACG | AGA | CTT | GTT | CGA | CTC | TGG | CCA | GGC | ACG | GCA | | | | |
| ▶ Leu | Asp | Lys | Arg | Glu | Val | Cys | Ser | Glu | Gln | Ala | Glu | Thr | Gly | Pro | Cys | Arg | | | | |
| AatII | | | | | | | | | | | | | | | | | | | | |
| GCA | ATG | ATC | TCC | CGC | TGG | TAC | TTT | GAC | GTC | ACT | GAA | GGT | AAG | TGC | GCT | CCA | | | | |
| CGT | TAC | TAG | AGG | GCG | ACC | ATG | AAA | CTG | CAG | TGA | CTT | CCA | TTC | ACG | CGA | GGT | | | | |
| ▶ Ala | Met | Ile | Ser | Arg | Trp | Tyr | Phe | Asp | Val | Thr | Glu | Gly | Lys | Cys | Ala | Pro | | | | |
| TTC | TTT | TAC | GGC | GGT | TGC | GGC | GGC | AAC | CGT | AAC | AAC | TTT | GAC | ACT | GAA | GAG | | | | |
| AAG | AAA | ATG | CCG | CCA | ACG | CCG | CCG | TTG | GCA | TTG | TTG | AAA | CTG | TGA | CTT | CTC | | | | |
| ▶ Phe | Phe | Tyr | Gly | Gly | Cys | Gly | Gly | Asn | Arg | Asn | Asn | Phe | Asp | Thr | Glu | Glu | | | | |
| BamHI | | | | | | | | HindIII | | | | | | | | | | | | |
| TAC | TGC | ATG | GCA | GTG | TGC | GGA | TCC | GCT | ATT | TA | | | | | | | | | | |
| ATG | ACG | TAC | CGT | CAC | ACG | CCT | AGG | CGA | TAA | ATT | CGA | | | | | | | | | |
| ▶ Tyr | Cys | Met | Ala | Val | Cys | Gly | Ser | Ala | Ile | | | | | | | | | | | |

5/59

FIG. 5



6 / 59

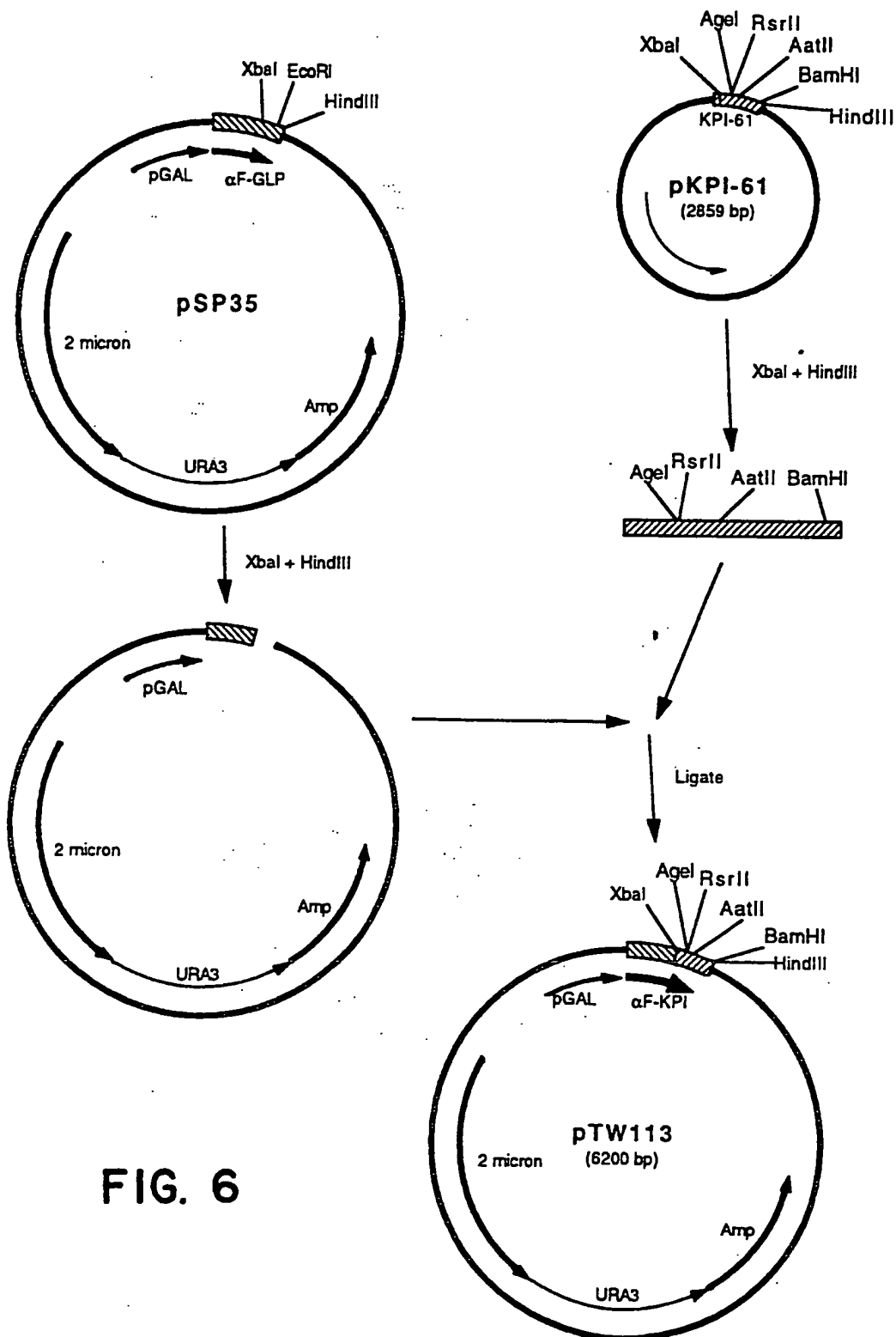
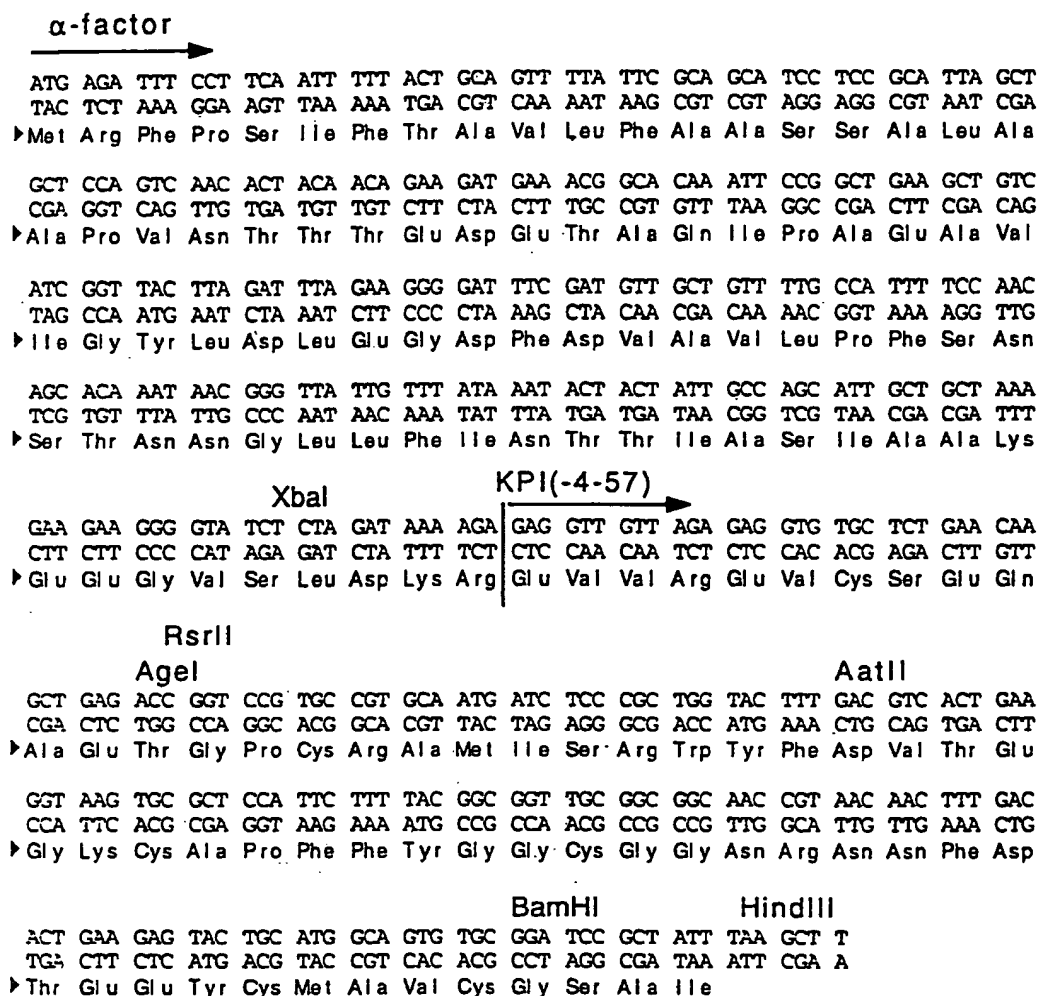


FIG. 6

7/59

FIG. 7



8/59

FIG. 8

KPI(-4-57)

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - Met - Ile - Ser - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

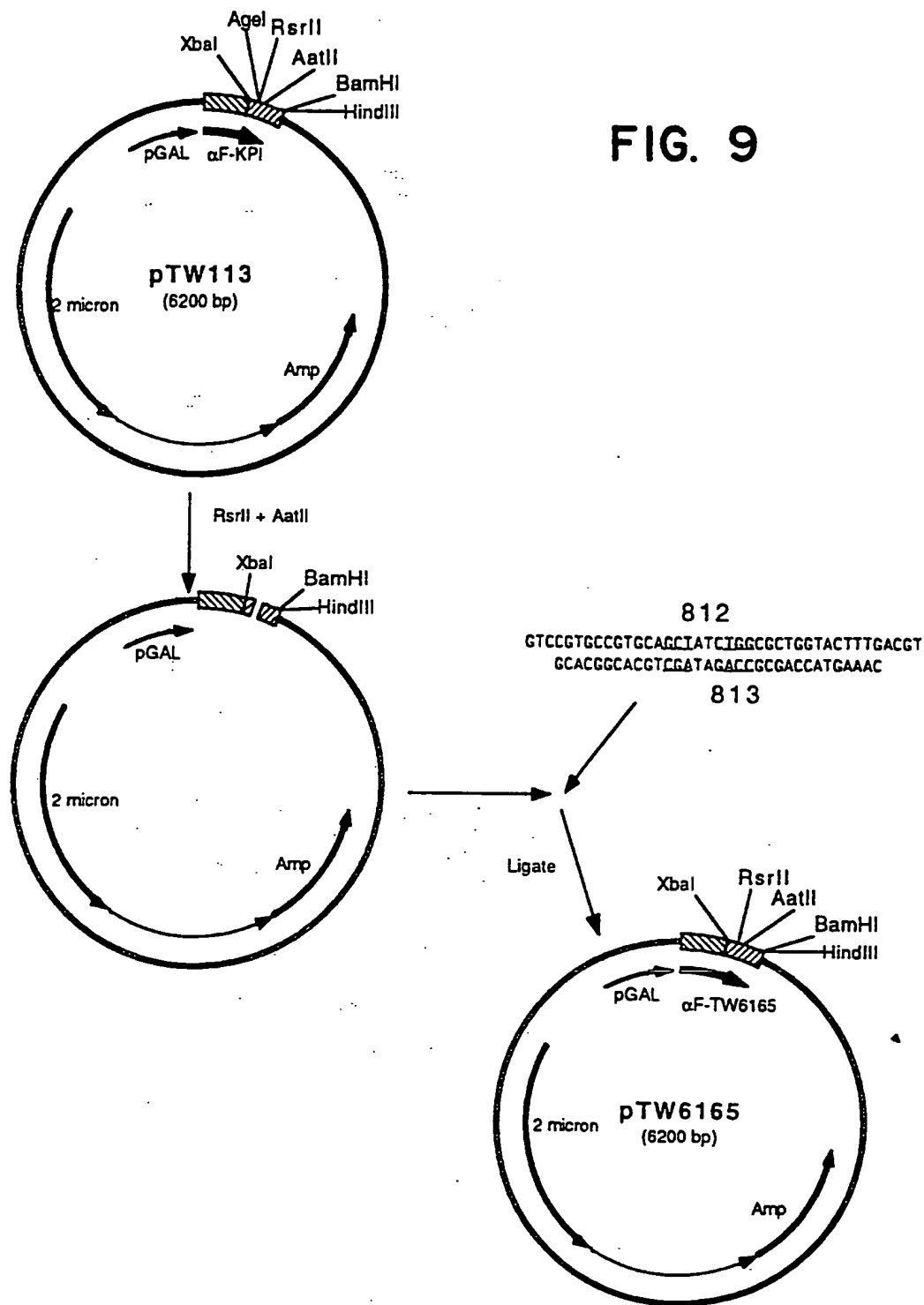
Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

9 / 59

FIG. 9



10 / 59

pTW 6165

FIG. 10

 α -factor

ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
 TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
 ▶ Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

 GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
 CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
 ▶ Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

 ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
 TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
 ▶ Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

 AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
 TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
 ▶ Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

XbaI

KPI(-4-57; M15A, S17W)

GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
 CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
 ▶ Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln

RsrII

AgeI

AatII

GCT GAG ACC GGT CCG TGC CGT GCA GCT ATC TGG CGC TGG TAC TTT GAC GTC ACT GAA
 CGA CTC TGG CCA GGC ACG GCA CGT CGA TAG ACC GCG ACC ATG AAA CTG CAG TGA CTT
 ▶ Ala Glu Thr Gly Pro Cys Arg Ala Ala Ile Trp Arg Trp Tyr Phe Asp Val Thr Glu

 GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
 CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
 ▶ Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

BamHI

HindIII

ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
 TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
 ▶ Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

11/59

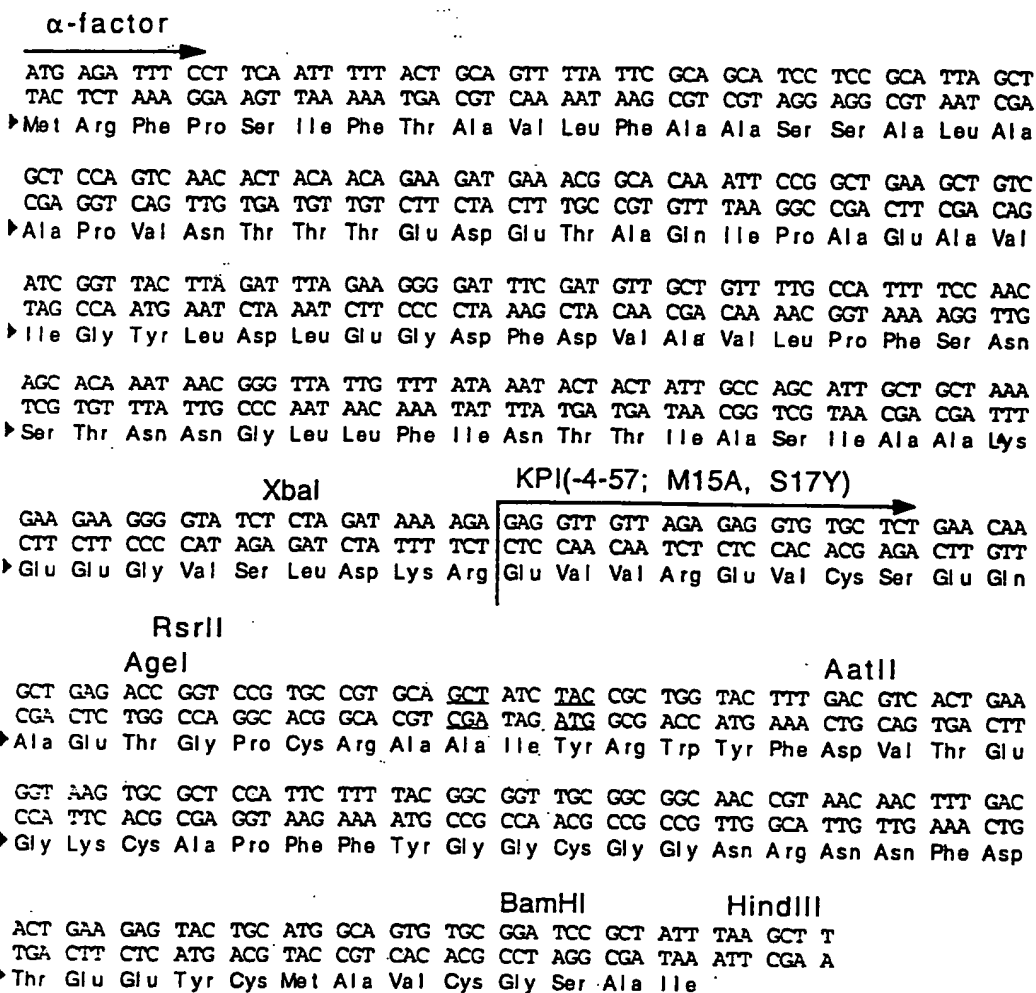
FIG. II

| | |
|--|--------------------------------|
| 812 GTCCGTGCCGTGCAGCTATCTGGCGCTGGTACTTTGACGT GCACGGCACGTCGATAGACCGGACCATGAAAC 813 | pTW6165 KPI(-4-57; M15A, S17F) |
| 814 GTCCGTGCCGTGCAGCTATCTACCGCTGGTACTTTGACGT GCACGGCACGTCGATAGATGGCGACCATGAAAC 815 | pTW6166 KPI(-4-57; M15A, S17Y) |
| 867 GTCCGTGCCGTGCATTGATCTTCCGCTGGTACTTTGACGT GCACGGCACGTAAC TAGAAGGCGACCATGAAAC 868 | pTW6175 KPI(-4-57; M15L, S17F) |
| 1493 GTCCGTGCCGTGCATTGATCTACCGCTGGTACTTTGACGT GCACGGCACGTAAC TAGATGGCGACCATGAAAC 1494 | pBG028 KPI(-4-57; M15L, S17Y) |
| 925 GTCCGTGCCGTGCAATGCACTTCCGCTGGTACTTTGACGT GCACGGCACGTTACGTGAAGGCGACCATGAAAC 926 | pTW6183 KPI(-4-57; I16H, S17F) |
| 927 GTCCGTGCCGTGCAATGCACTACCGCTGGTACTTTGACGT GCACGGCACGTTACGTGATGGCGACCATGAAAC 928 | pTW6184 KPI(-4-57; I16H, S17Y) |
| 929 GTCCGTGCCGTGCAATGCACTGGCGCTGGTACTTTGACGT GCACGGCACGTTACGTGACCGGACCATGAAAC 930 | pTW6185 KPI(-4-57; I16H, S17W) |
| 863 GTCCGTGCCGTGCAGCTCACTCCCGCTGGTACTTTGACGT GCACGGCACGTCGATGAGGGCGACCATGAAAC 864 | pTW6173 KPI(-4-57; M15A, I16H) |
| 865 GTCCGTGCCGTGCATTGCACTCCCGCTGGTACTTTGACGT GCACGGCACGTAACGTGAGGGCGACCATGAAAC 866 | pTW6174 KPI(-4-57; M15L, I16H) |

12 / 59

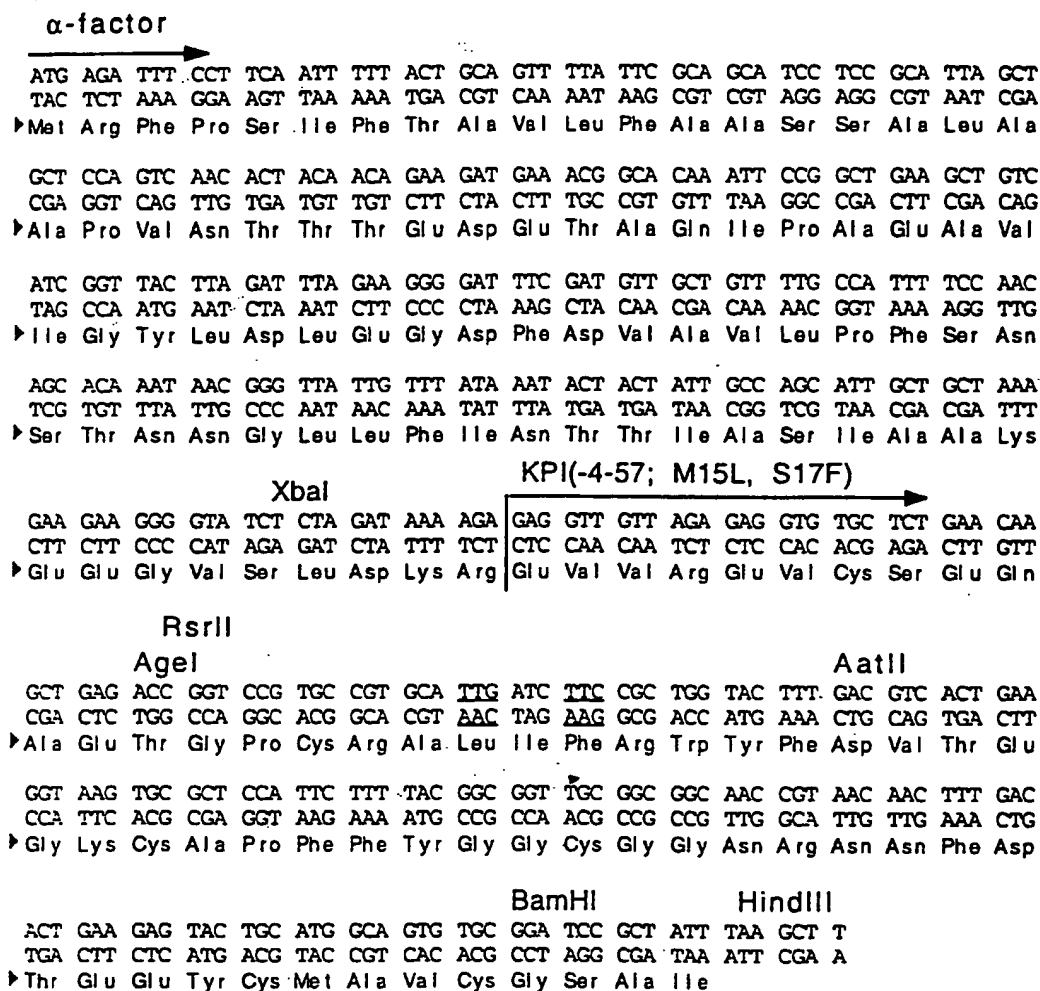
pTW 6166

FIG. 12



13/59

FIG. 13



14 / 59

FIG. 14

α-factor

ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
▶ Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
▶ Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
▶ Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
▶ Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

XbaI **KPI(-4-57; M15L, S17Y)**

GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
▶ Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln

RsrII

AgeI **AatII**

GCT GAG ACC GGT CCG TGC CGT GCA TTG ATC TAC CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT AAC TAG ATG GCG ACC ATG AAA CTG CAG TGA CTT
▶ Ala Glu Thr Gly Pro Cys Arg Ala Leu Ile Tyr Arg Trp Tyr Phe Asp Val Thr Glu

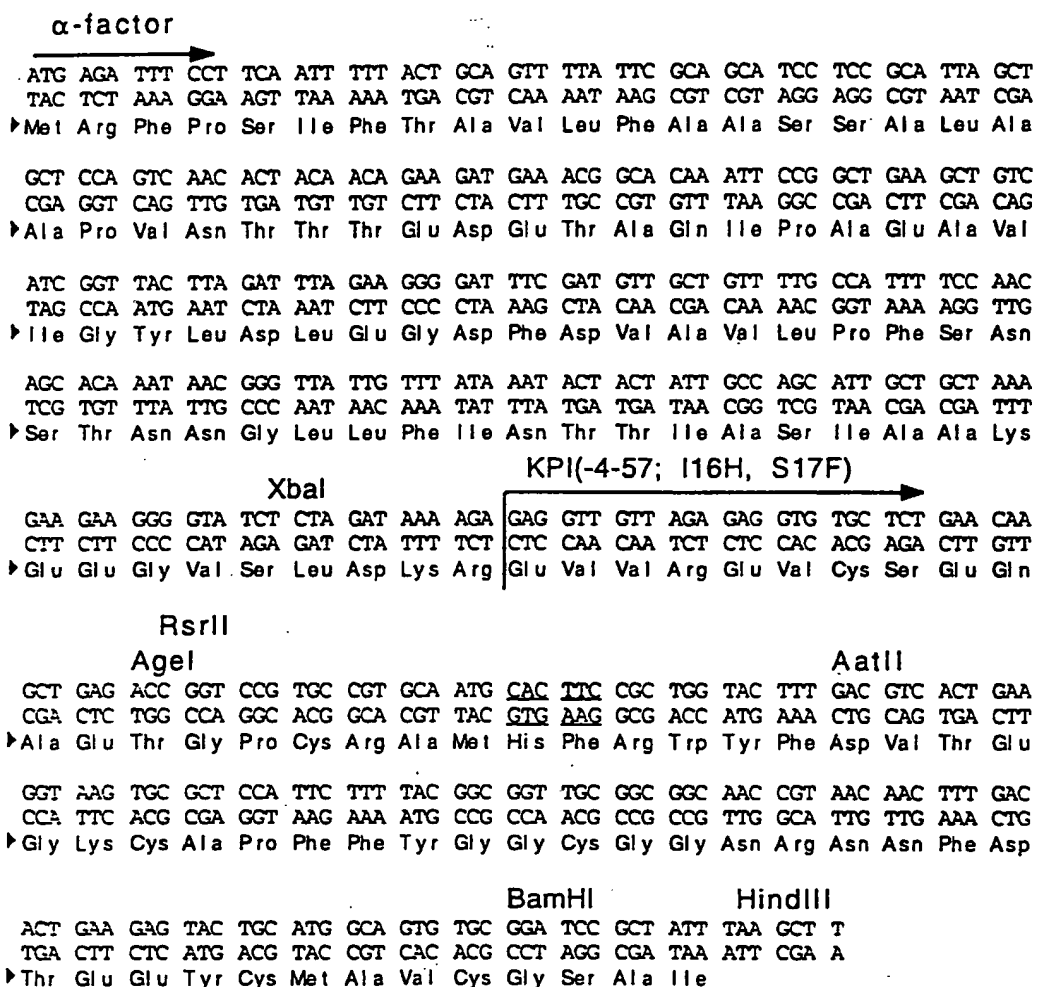
GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
▶ Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

BamHI **HindIII**

ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
▶ Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

15/59

FIG. 15



16/59

FIG. 16

 α -factor

ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
 TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
 ▶ Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
 CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
 ▶ Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
 TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
 ▶ Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
 TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
 ▶ Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

XbaI

KPI(-4-57; I16H, S17Y)

GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
 CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
 ▶ Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln

RsrII

AgeI

AatII

GCT GAG ACC GGT CCG TGC CGT GCA ATG CAC TAC CGC TGG TAC TTT GAC GTC ACT GAA
 CGA CTC TGG CCA GGC ACG GCA CGT TAC GTG ATG GCG ACC ATG AAA CTG CAG TGA CTT
 ▶ Ala Glu Thr Gly Pro Cys Arg Ala Met His Tyr Arg Trp Tyr Phe Asp Val Thr Glu

GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
 CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
 ▶ Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

BamHI

HindIII

ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
 TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
 ▶ Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

17 / 59

FIG. 17

α-factor

ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
▶ Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
▶ Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
▶ Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
▶ Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

XbaI

GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
▶ Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln

RsrII

AgeI

GCT GAG ACC GGT CCG TGC CGT GCA ATG CAC TGG CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT TAC GTG ACC GCG ACC ATG AAA CTG CAG TGA CTT
▶ Ala Glu Thr Gly Pro Cys Arg Ala Met His Trp Arg Trp Tyr Phe Asp Val Thr Glu

AatII

GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
▶ Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp



BamHI

ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
▶ Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

HindIII

18 / 59

FIG. 18

| | | | | | | | | | | | | | | | | | | | |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|------------------------|-----|-----|-----|-----|-----|-----|-----|-----|--|
| α -factor | | | | | | | | | | | | | | | | | | | |
|  | | | | | | | | | | | | | | | | | | | |
| ATG | AGA | TTT | CCT | TCA | ATT | TTT | ACT | GCA | GTT | TTA | TTC | GCA | GCA | TCC | TCC | GCA | TTA | GCT | |
| TAC | TCT | AAA | GGA | AGT | TAA | AAA | TGA | CGT | CAA | AAT | AAG | CGT | CGT | AGG | AGG | CGT | AAT | CGA | |
| Met | Arg | Phe | Pro | Ser | Ile | Phe | Thr | Ala | Val | Leu | Phe | Ala | Ala | Ser | Ser | Ala | Leu | Ala | |
| | | | | | | | | | | | | | | | | | | | |
| GCT | CCA | GTC | AAC | ACT | ACA | ACA | GAA | GAT | GAA | ACG | GCA | CAA | ATT | CCG | GCT | GAA | GCT | GTC | |
| CGA | GGT | CAG | TTG | TGA | TGT | TGT | CTT | CTA | CTT | TGC | CGT | GTT | TAA | GGC | CGA | CTT | CGA | CAG | |
| Ala | Pro | Val | Asn | Thr | Thr | Thr | Glu | Asp | Glu | Thr | Ala | Gln | Ile | Pro | Ala | Glu | Ala | Val | |
| | | | | | | | | | | | | | | | | | | | |
| ATC | GGT | TAC | TTA | GAT | TTA | GAA | GGG | GAT | TTC | GAT | GTT | GCT | GTT | TTG | CCA | TTT | TCC | AAC | |
| TAG | CCA | ATG | AAT | CTA | AAT | CTT | CCC | CTA | AAG | CTA | CAA | CGA | CAA | AAC | GGT | AAA | AGG | TTG | |
| Ile | Gly | Tyr | Leu | Asp | Leu | Glu | Gly | Asp | Phe | Asp | Val | Ala | Val | Leu | Pro | Phe | Ser | Asn | |
| | | | | | | | | | | | | | | | | | | | |
| AGC | ACA | AAT | AAC | GGG | TTA | TTG | TTT | ATA | AAT | ACT | ACT | ATT | GCC | AGC | ATT | GCT | GCT | AAA | |
| TCG | TGT | TTA | TTG | CCC | AAT | AAC | AAA | TAT | TTA | TGA | TGA | TAA | CGG | TCG | TAA | CGA | CGA | TTT | |
| Ser | Thr | Asn | Asn | Gly | Leu | Leu | Phe | Ile | Asn | Thr | Thr | Ile | Ala | Ser | Ile | Ala | Ala | Lys | |
| | | | | | | | | | | | | | | | | | | | |
| XbaI | | | | | | | | | | KPI(-4-57; M15A, I16H) | | | | | | | | | |
|  | | | | | | | | | | | | | | | | | | | |
| GAA | GAA | GGG | GTA | TCT | CTA | GAT | AAA | AGA | GAG | GTT | GTT | AGA | GAG | GTG | TGC | TCT | GAA | CAA | |
| CTT | CTT | CCC | CAT | AGA | GAT | CTA | TTT | TCT | CTC | CAA | CAA | TCT | CTC | CAC | ACG | AGA | CTT | GTT | |
| Glu | Glu | Gly | Val | Ser | Leu | Asp | Lys | Arg | Glu | Val | Val | Arg | Glu | Val | Cys | Ser | Glu | Gln | |
| | | | | | | | | | | | | | | | | | | | |
| RsrII | | | | | | | | | | AatII | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | |
| AgeI | | | | | | | | | | | | | | | | | | | |
| GCT | GAG | ACC | GGT | CCG | TGC | CGT | GCA | GCT | CAC | TCC | CGC | TGG | TAC | TTT | GAC | GTC | ACT | GAA | |
| CGA | CTC | TGG | CCA | GGC | ACG | GCA | CGT | CGA | GTG | AGG | GCG | ACC | ATG | AAA | CTG | CAG | TGA | CTT | |
| Ala | Glu | Thr | Gly | Pro | Cys | Arg | Ala | Ala | His | Ser | Arg | Trp | Tyr | Phe | Asp | Val | Thr | Glu | |
| | | | | | | | | | | | | | | | | | | | |
| GGT | AAG | TGC | GCT | CCA | TTC | TTT | TAC | GGC | GGT | TGC | GGC | GGC | AAC | CGT | AAC | AAC | TTT | GAC | |
| CCA | TTC | ACG | CGA | GGT | AAG | AAA | ATG | CCG | CCA | ACG | CCG | CCG | TTG | GCA | TTG | TTG | AAA | CTG | |
| Gly | Lys | Cys | Ala | Pro | Phe | Phe | Tyr | Gly | Gly | Cys | Gly | Gly | Asn | Arg | Asn | Asn | Phe | Asp | |
| | | | | | | | | | | | | | | | | | | | |
| BamHI | | | | | | | | | | HindIII | | | | | | | | | |
| ACT | GAA | GAG | TAC | TGC | ATG | GCA | GTG | TGC | GGA | TCC | GCT | ATT | TAA | GCT | T | | | | |
| TGA | CTT | CTC | ATG | ACG | TAC | CGT | CAC | ACG | CCT | AGG | CGA | TAA | ATT | CGA | A | | | | |
| Thr | Glu | Glu | Tyr | Cys | Met | Ala | Val | Cys | Gly | Ser | Ala | Ile | | | | | | | |

19/59

FIG. 19

| α-factor | | | | | | | | | | | | | | | | | | | |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|------------------------|-----|-----|-----|-----|-----|-----|-----|-----|--|
| ATG | AGA | TTT | CCT | TCA | ATT | TTT | ACT | GCA | GTT | TTA | TTC | GCA | GCA | TCC | TCC | GCA | TTA | GCT | |
| TAC | TCT | AAA | GGA | AGT | TAA | AAA | TGA | CGT | CAA | AAT | AAG | CGT | CGT | AGG | AGG | CGT | AAT | CGA | |
| Met | Arg | Phe | Pro | Ser | Ile | Phe | Thr | Ala | Val | Leu | Phe | Ala | Ala | Ser | Ser | Ala | Leu | Ala | |
| | | | | | | | | | | | | | | | | | | | |
| GCT | CCA | GTC | AAC | ACT | ACA | ACA | GAA | GAT | GAA | ACG | GCA | CAA | ATT | CCG | GCT | GAA | GCT | GTC | |
| CGA | GGT | CAG | TTG | TGA | TGT | TGT | CTT | CTA | CTT | TGC | CGT | GTT | TAA | GGC | CGA | CTT | CGA | CAG | |
| Ala | Pro | Val | Asn | Thr | Thr | Thr | Glu | Asp | Glu | Thr | Ala | Gln | Ile | Pro | Ala | Glu | Ala | Val | |
| | | | | | | | | | | | | | | | | | | | |
| ATC | GGT | TAC | TTA | GAT | TTA | GAA | GGG | GAT | TTC | GAT | GTT | GCT | GTT | TTG | CCA | TTT | TCC | AAC | |
| TAG | CCA | ATG | AAT | CTA | AAT | CTT | CCC | CTA | AAG | CTA | CAA | CGA | CAA | AAC | GGT | AAA | AGG | TTG | |
| Ile | Gly | Tyr | Leu | Asp | Leu | Glu | Gly | Asp | Phe | Asp | Val | Ala | Val | Leu | Pro | Phe | Ser | Asn | |
| | | | | | | | | | | | | | | | | | | | |
| AGC | ACA | AAT | AAC | GGG | TTA | TTG | TTT | ATA | AAT | ACT | ACT | ATT | GCC | AGC | ATT | GCT | GCT | AAA | |
| TCG | TGT | TTA | TTG | CCC | AAT | AAC | AAA | TAT | TTA | TGA | TGA | TAA | CGG | TCG | TAA | CGA | CGA | TTT | |
| Ser | Thr | Asn | Asn | Gly | Leu | Leu | Phe | Ile | Asn | Thr | Thr | Ile | Ala | Ser | Ile | Ala | Ala | Lys | |
| | | | | | | | | | | | | | | | | | | | |
| XbaI | | | | | | | | | | KPI(-4-57; M15L, I16H) | | | | | | | | | |
| GAA | GAA | GGG | GTA | TCT | CTA | GAT | AAA | AGA | GAG | GTT | GTT | AGA | GAG | GTG | TGC | TCT | GAA | CAA | |
| CTT | CTT | CCC | CAT | AGA | GAT | CTA | TTT | TCT | CTC | CAA | CAA | TCT | CTC | CAC | ACG | AGA | CTT | GTT | |
| Glu | Glu | Gly | Val | Ser | Leu | Asp | Lys | Arg | Glu | Val | Val | Arg | Glu | Val | Cys | Ser | Glu | Gln | |
| | | | | | | | | | | | | | | | | | | | |
| RsrII | | | | | | | | | | AatII | | | | | | | | | |
| AgeI | | | | | | | | | | | | | | | | | | | |
| GCT | GAG | ACC | GGT | CCG | TGC | CGT | GCA | TTG | CAC | TCC | CGC | TGG | TAC | TTT | GAC | GTC | ACT | GAA | |
| CGA | CTC | TGG | CCA | GGC | ACG | GCA | CGT | AAC | GTG | AGG | GCG | ACC | ATG | AAA | CTG | CAG | TGA | CTT | |
| Ala | Glu | Thr | Gly | Pro | Cys | Arg | Ala | Leu | His | Ser | Arg | Trp | Tyr | Phe | Asp | Val | Thr | Glu | |
| | | | | | | | | | | | | | | | | | | | |
| GGT | AAG | TGC | GCT | CCA | TTC | TTT | TAC | GGC | GGT | TGC | GGC | GGC | AAC | CGT | AAC | AAC | TTT | GAC | |
| CCA | TTC | ACG | CGA | GGT | AAG | AAA | ATG | CCG | CCA | ACG | CCG | CCG | TTG | GCA | TTG | TTG | AAA | CTG | |
| Gly | Lys | Cys | Ala | Pro | Phe | Phe | Tyr | Gly | Gly | Cys | Gly | Gly | Asn | Arg | Asn | Asn | Phe | Asp | |
| | | | | | | | | | | | | | | | | | | | |
| BamHI | | | | | | | | | | HindIII | | | | | | | | | |
| ACT | GAA | GAG | TAC | TGC | ATG | GCA | GTG | TGC | GGA | TCC | GCT | ATT | TAA | GCT | T | | | | |
| TGA | CTT | CTC | ATG | ACG | TAC | CGT | CAC | ACG | CCT | AGG | CGA | TAA | ATT | CGA | A | | | | |
| Thr | Glu | Glu | Tyr | Cys | Met | Ala | Val | Cys | Gly | Ser | Ala | Ile | | | | | | | |

20/59

FIG. 20

KPI(-4-57; M15A, S17W) TW6165

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - Ala - Ile - Trp - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

21/59

FIG. 21

KPI(-4-57; M15A, S17Y) TW6166

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - ~~Ala~~ - Ile - ~~Tyr~~ - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

22 / 59

FIG. 22

KPI(-4-57; M15L, S17F) TW6175

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - Leu - Ile - Phe - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

23 / 59

FIG. 23

KPI(-4-57; M15L, S17Y) BG028

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - Leu - Ile - Tyr - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

24 / 59

FIG. 24

KPI(-4-57; I16H, S17F) TW6183

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - Met - His - Phe - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

25 / 59

FIG. 25

KPI(-4-57; I16H, S17Y) TW6184

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - Met - His - Tyr - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

26 / 59

FIG. 26

KPI(-4-57; I16H, S17W) TW6185

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - Met - His - Trp - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

27/59

FIG. 27

KPI(-4-57; M15A, S17F) DD185

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - Ala - Ile - Phe - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

28 / 59

FIG. 28

KPI(-4-57; M15A, I16H) TW6173

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - Ala - His - Ser - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

29/59

FIG. 29

KPI(-4-57; M15L, I16H) TW6174

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - Leu - His - Ser - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

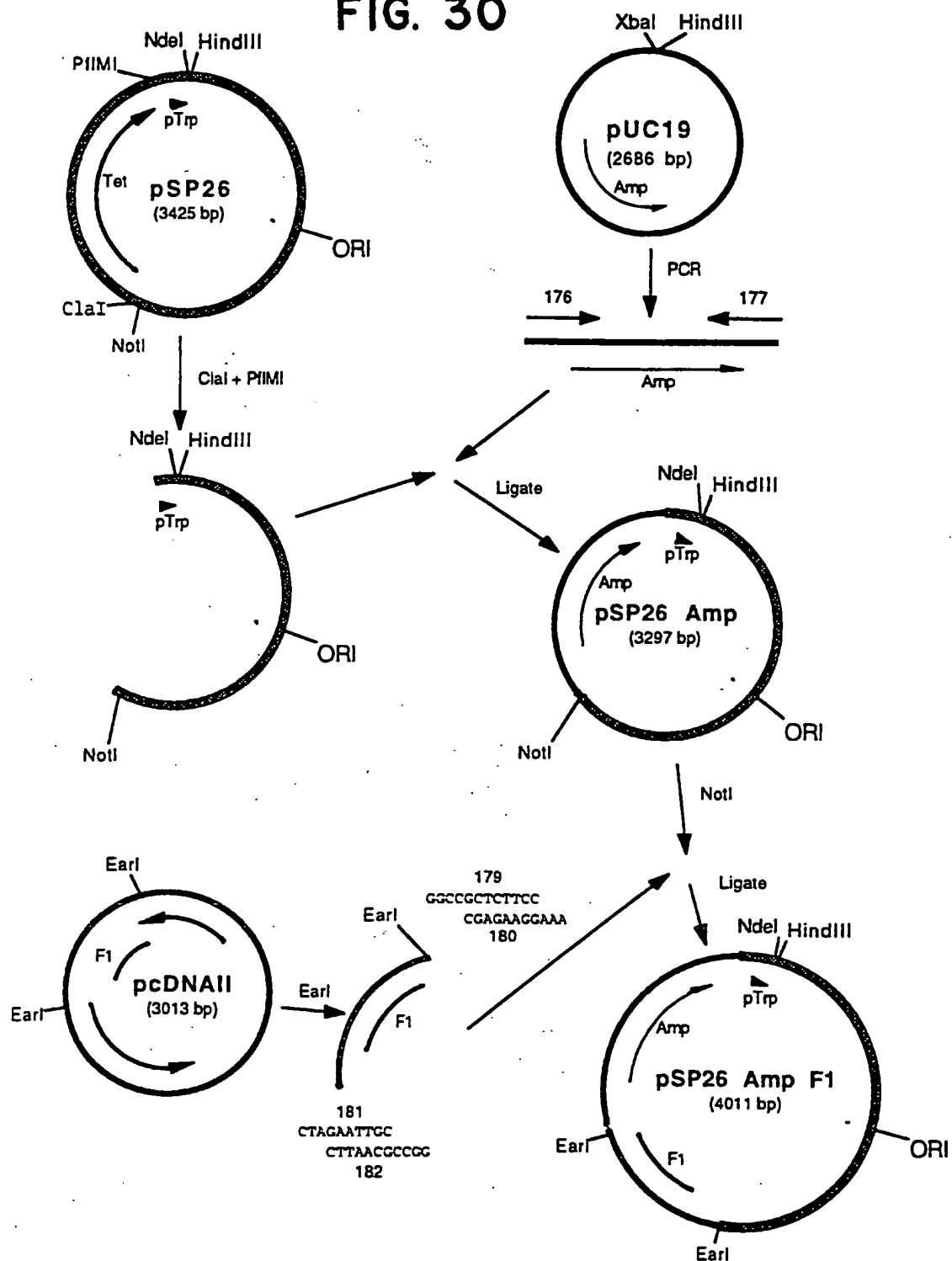
Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

30 / 59

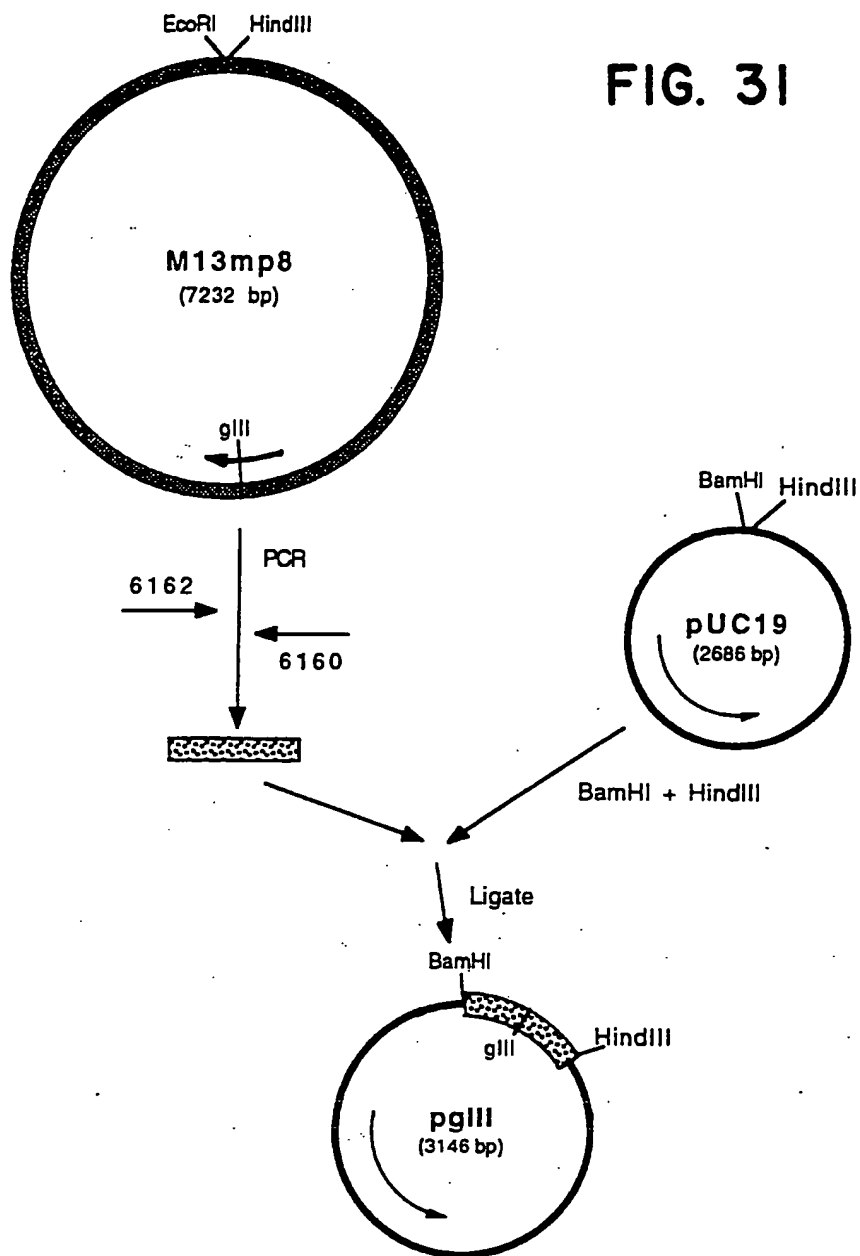
FIG. 30



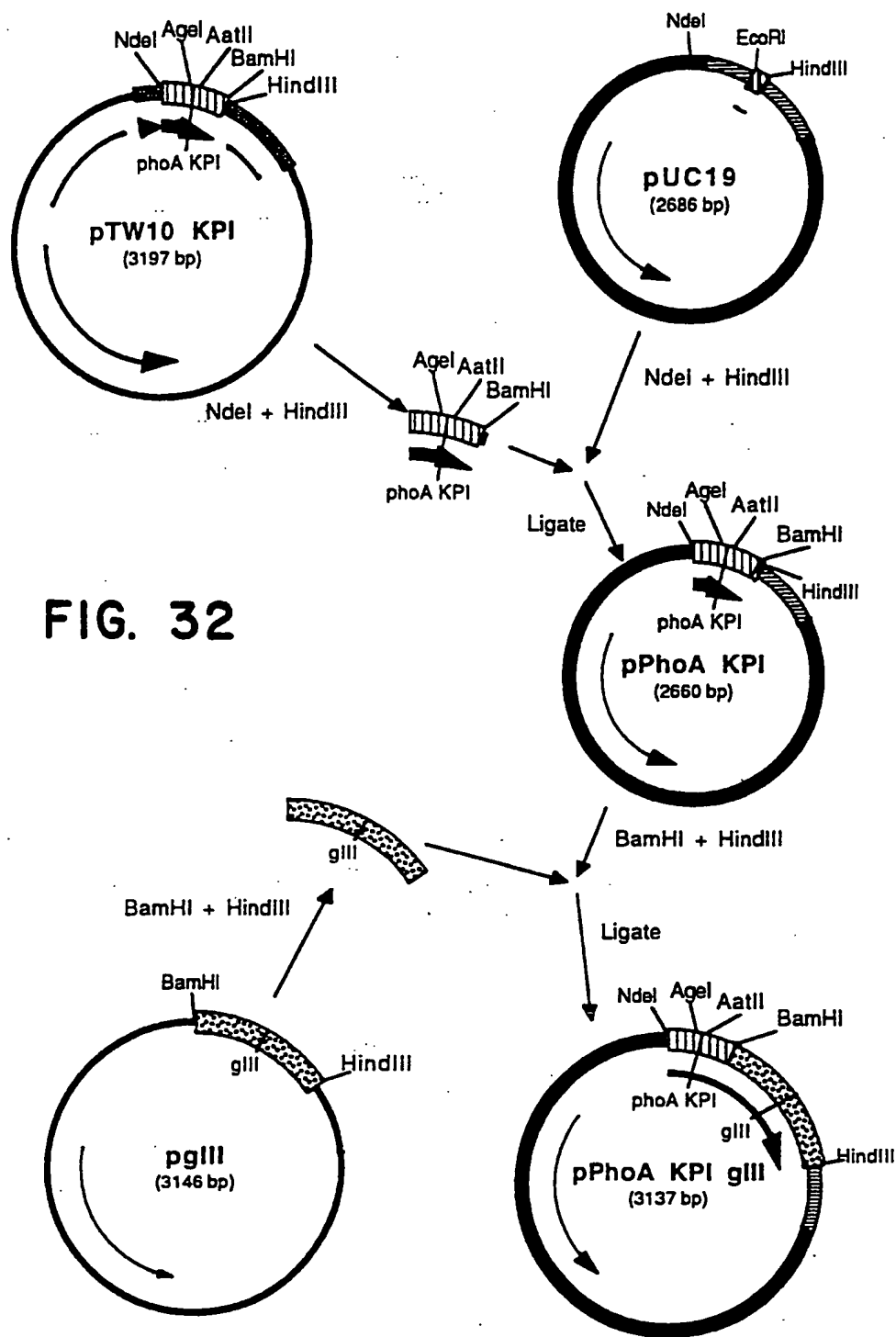
SUBSTITUTE SHEET (RULE 26)

31/59

FIG. 31

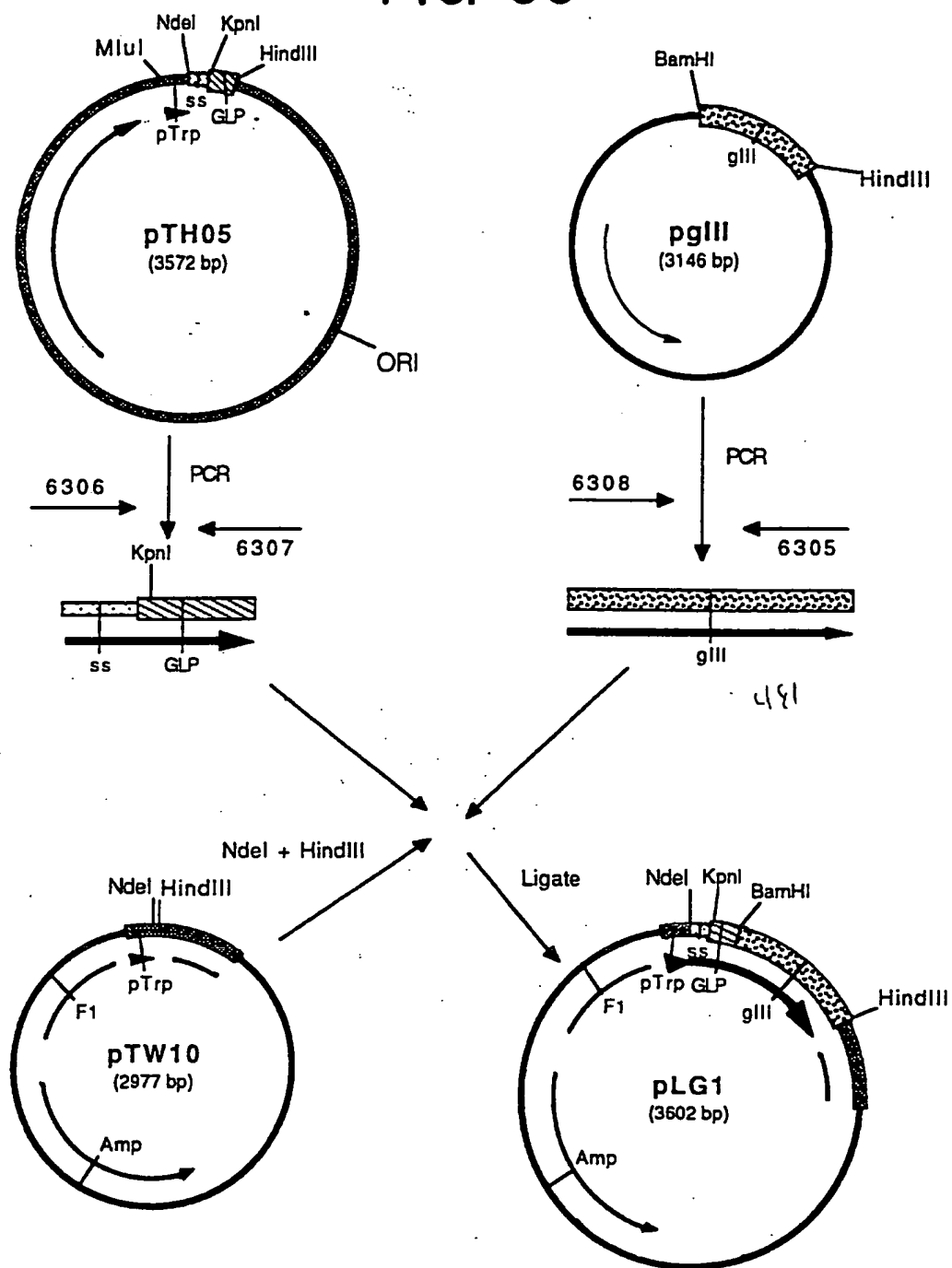


32 / 59



33 / 59

FIG. 33



SUBSTITUTE SHEET (RULE 26)

34 / 59

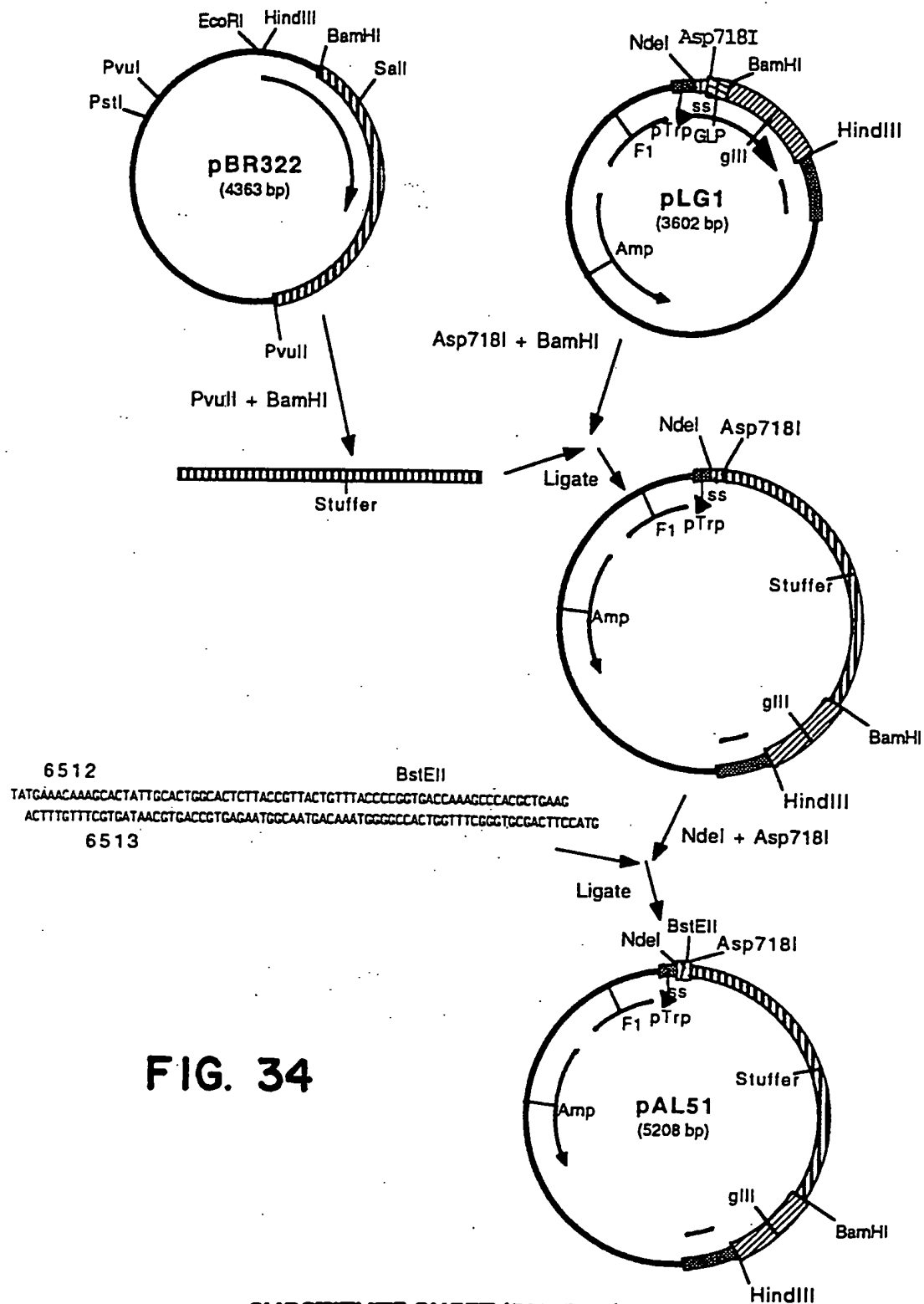
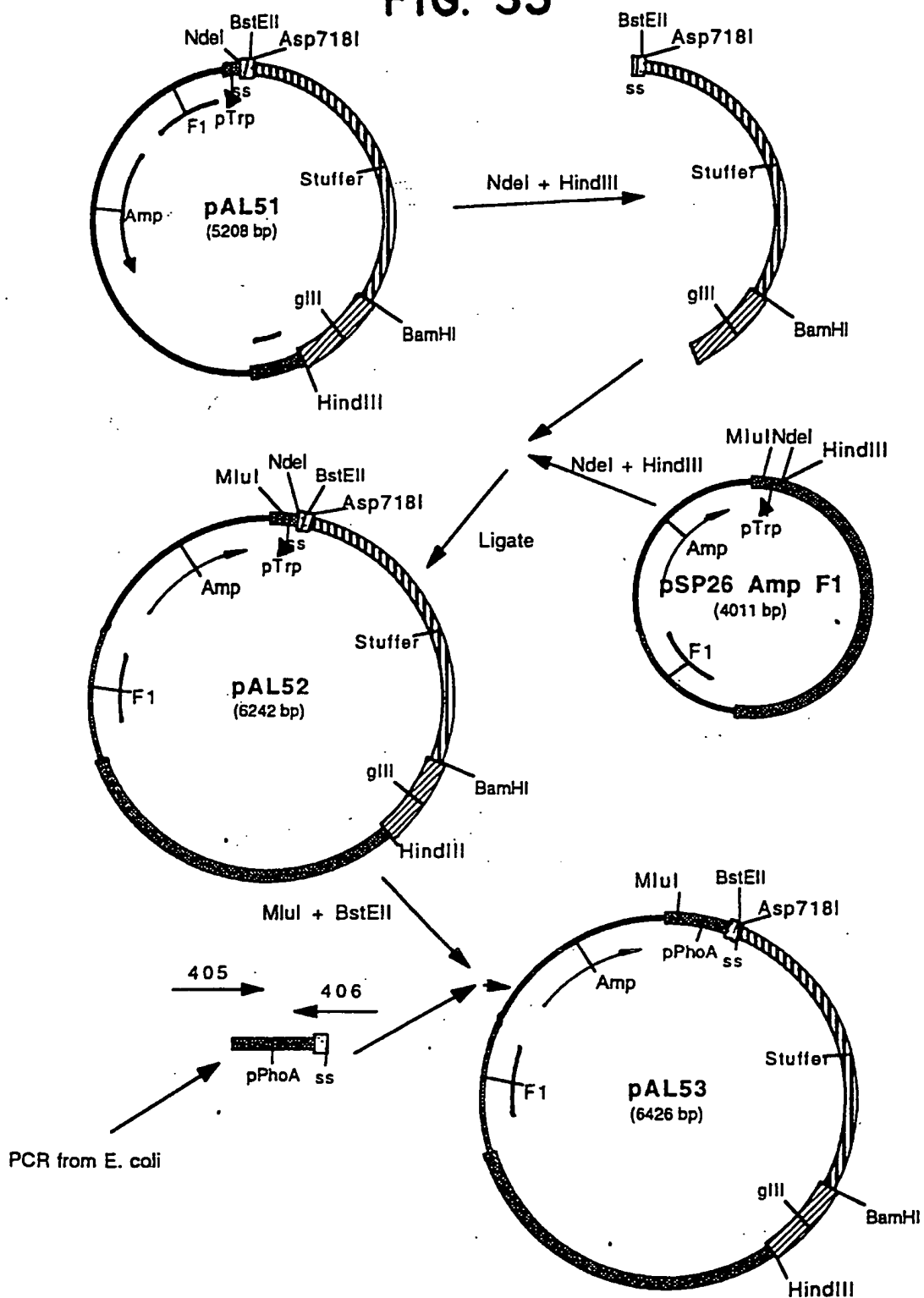


FIG. 34

35 / 59

FIG. 35



SUBSTITUTE SHEET (RULE 26)

36 / 59

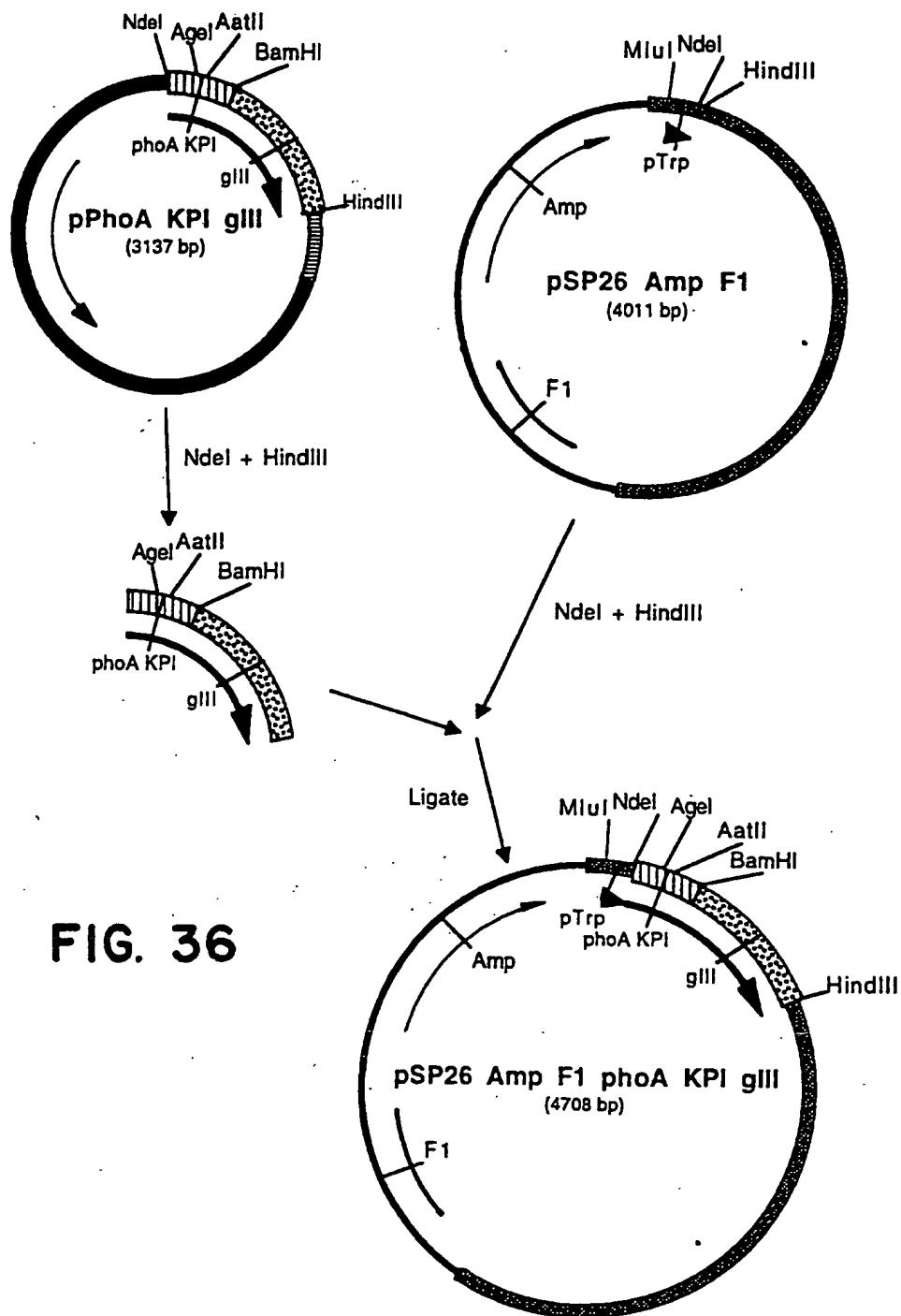
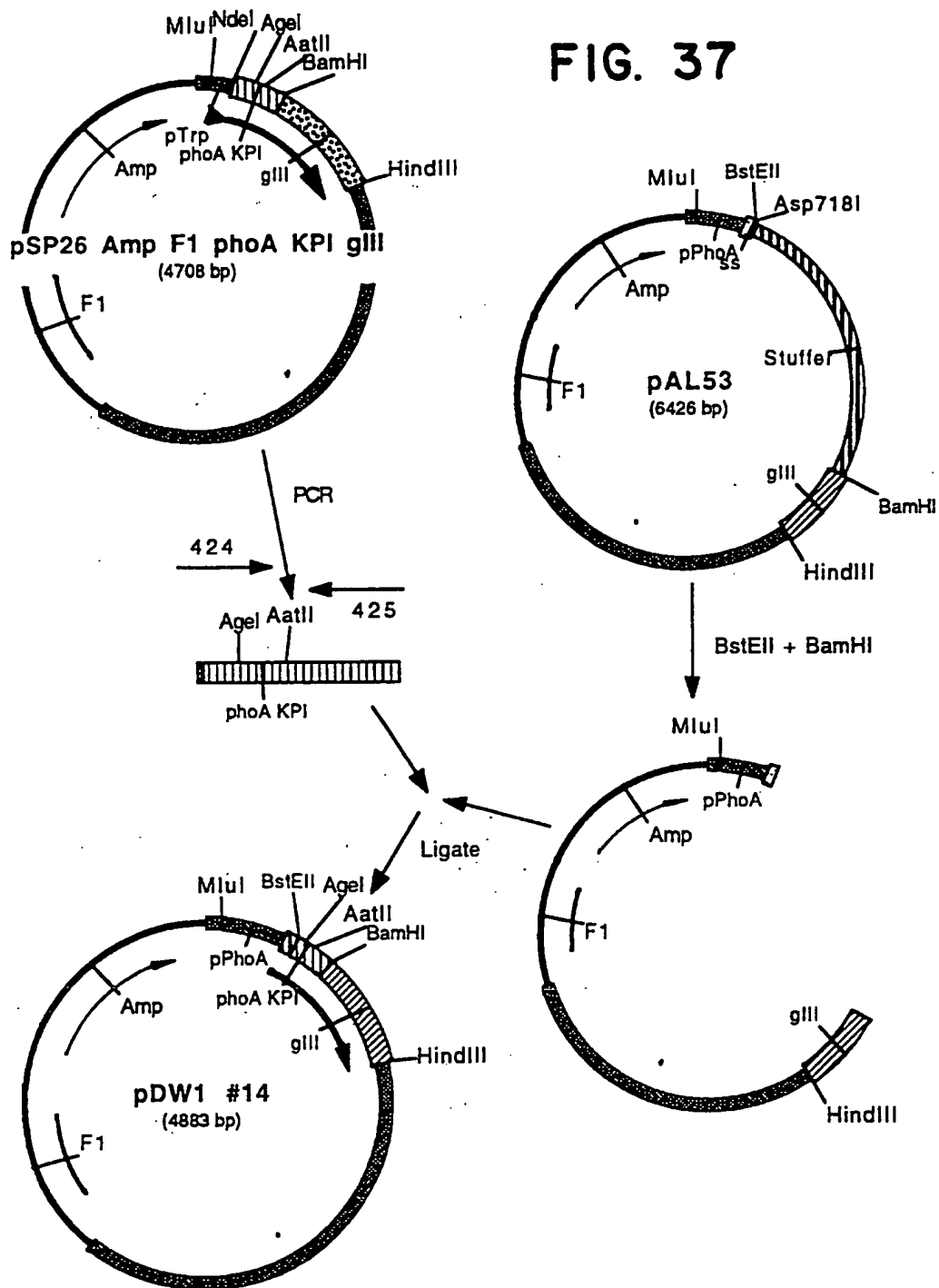


FIG. 36

37 / 59

FIG. 37



38 / 59

FIG. 38

phoA signal →

GTG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCG GTG ACC AAA
 ▶ Val Lys Gln Ser Thr Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys

BstEII

KPI (1-55) → Agel

GCC GAG GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT GCA ATG ATC TCC CGC TGG
 ▶ Ala Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met Ile Ser Arg Trp

AatII

TAC TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC
 ▶ Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn

BamHI gIII →

CGT AAC AAC TTT GAC ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GGT GGT GGC TCT
 ▶ Arg Asn Asn Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Gly Gly Gly Ser

GGT TCC GGT GAT TTT GAT TAT GAA AAG ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA
 ▶ Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu

AAT GCC GAT GAA AAC GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT ACT
 ▶ Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr

GAT TAC GGT GCT GCT ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC CTT GCT AAT GGT AAT
 ▶ Asp Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn

GGT GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC CAA ATG GCT CAA GTC GGT GAC GGT GAT
 ▶ Gly Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp

AAT TCA CCT TTA ATG AAT AAT TTC CGT CAA TAT TTA CCT TCC CTC CCT CAA TCG GTT GAA
 ▶ Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu

TGT CGC CCT TTT GTC TTT GGC GCT GGT AAA CCA TAC GAA TTT TCT ATT GAT TGT GAC AAA
 ▶ Cys Arg Pro Phe Val Phe Gly Ala Gly Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys

ATA AAC TTA TTC CGT GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT ATG TAT GTA
 ▶ Ile Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val

TTT TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT TAA TA
 ▶ Phe Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser ...

39 / 59

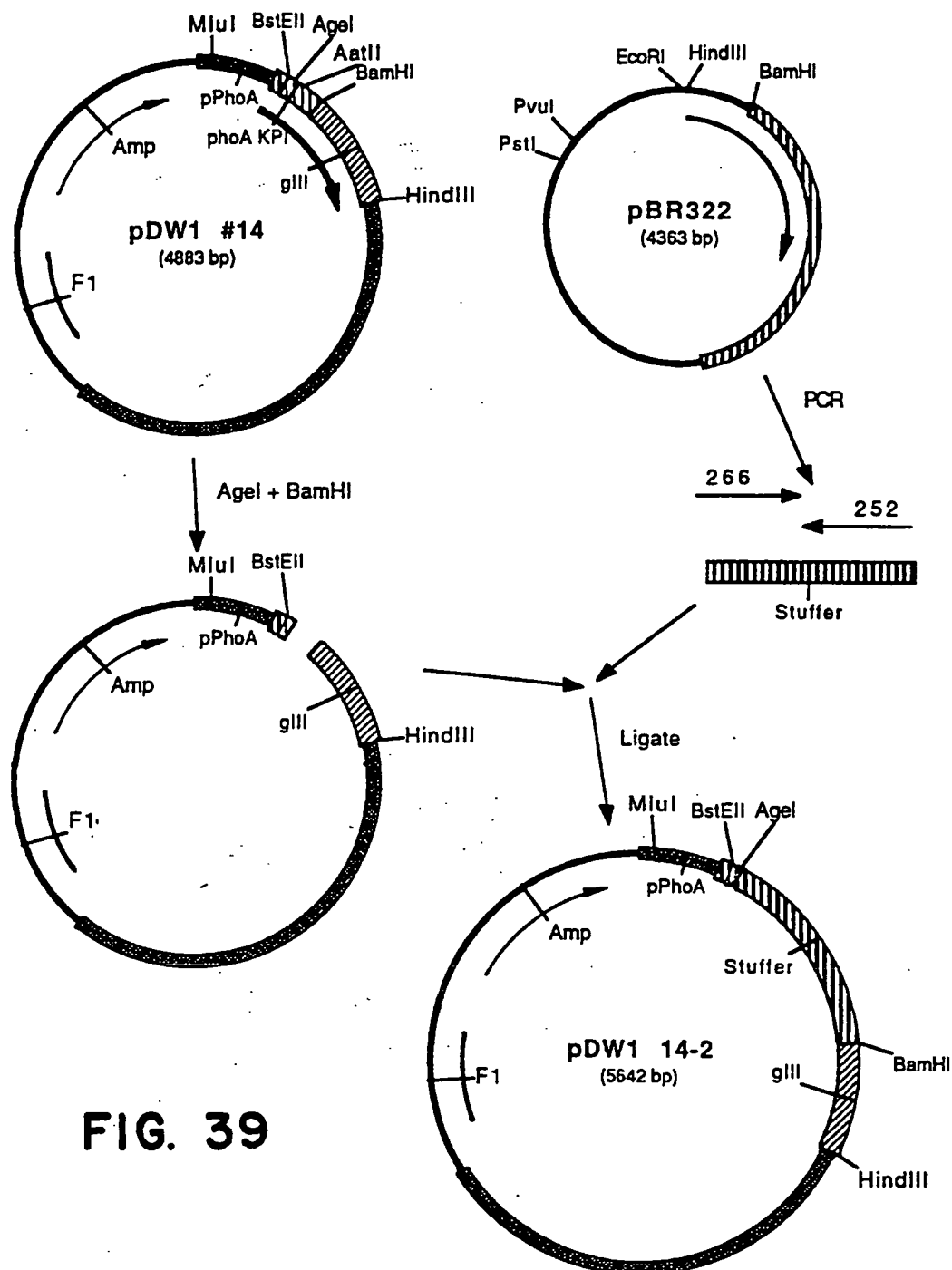
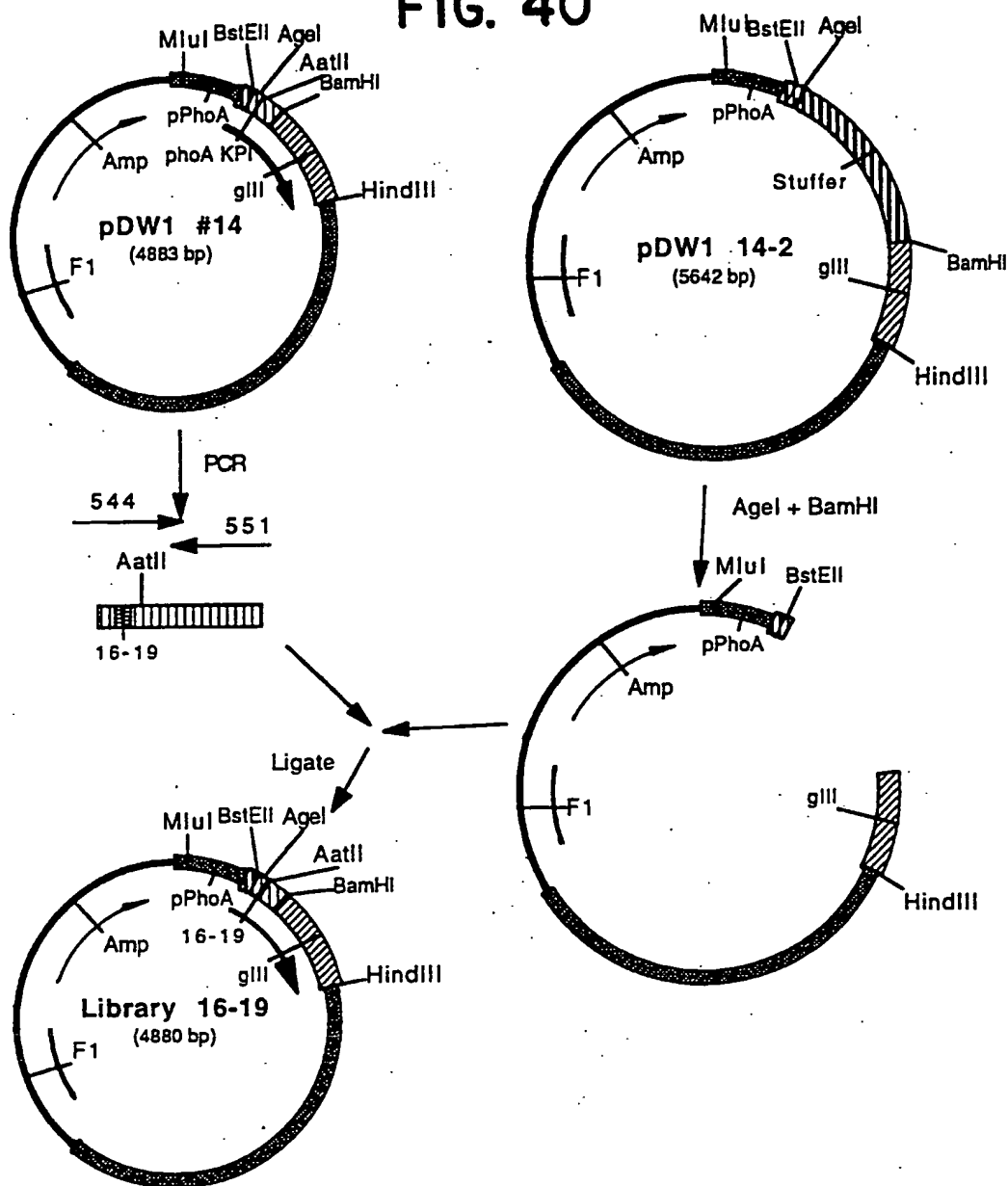


FIG. 39

40 / 59

FIG. 40



41/ 59

FIG. 4I

phoA signal →

GTG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCG GTG ACC AAA
 ▶ Val Lys Gln Ser Thr Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys

KPI (1-55; 16-19)

GCC GAG GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT NNS NNS NNS NNS TGG TAC
 ▶ Ala Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg ??? ??? ??? ??? Trp Tyr

Agel

16-19

AatII

TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT
 ▶ Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg

BamHI

gIII →

AAC AAC TTT GAC ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GGT GGT GGC TCT GGT
 ▶ Asn Asn Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Gly Gly Gly Ser Gly

TCC GGT GAT TTT GAT TAT GAA AAG ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA AAT
 ▶ Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu Asn

GCC GAT GAA AAC GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT ACT GAT
 ▶ Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr Asp

TAC GGT GCT GCT ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC CTT GCT AAT GGT AAT GGT
 ▶ Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn Gly

gIII

GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC CAA ATG GCT CAA GTC GGT GAC GGT GAT AAT
 ▶ Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp Asn

TCA CCT TTA ATG AAT AAT TTC CGT CAA TAT TTA CCT TCC CTC CCT CAA TCG GTT GAA TGT
 ▶ Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu Cys

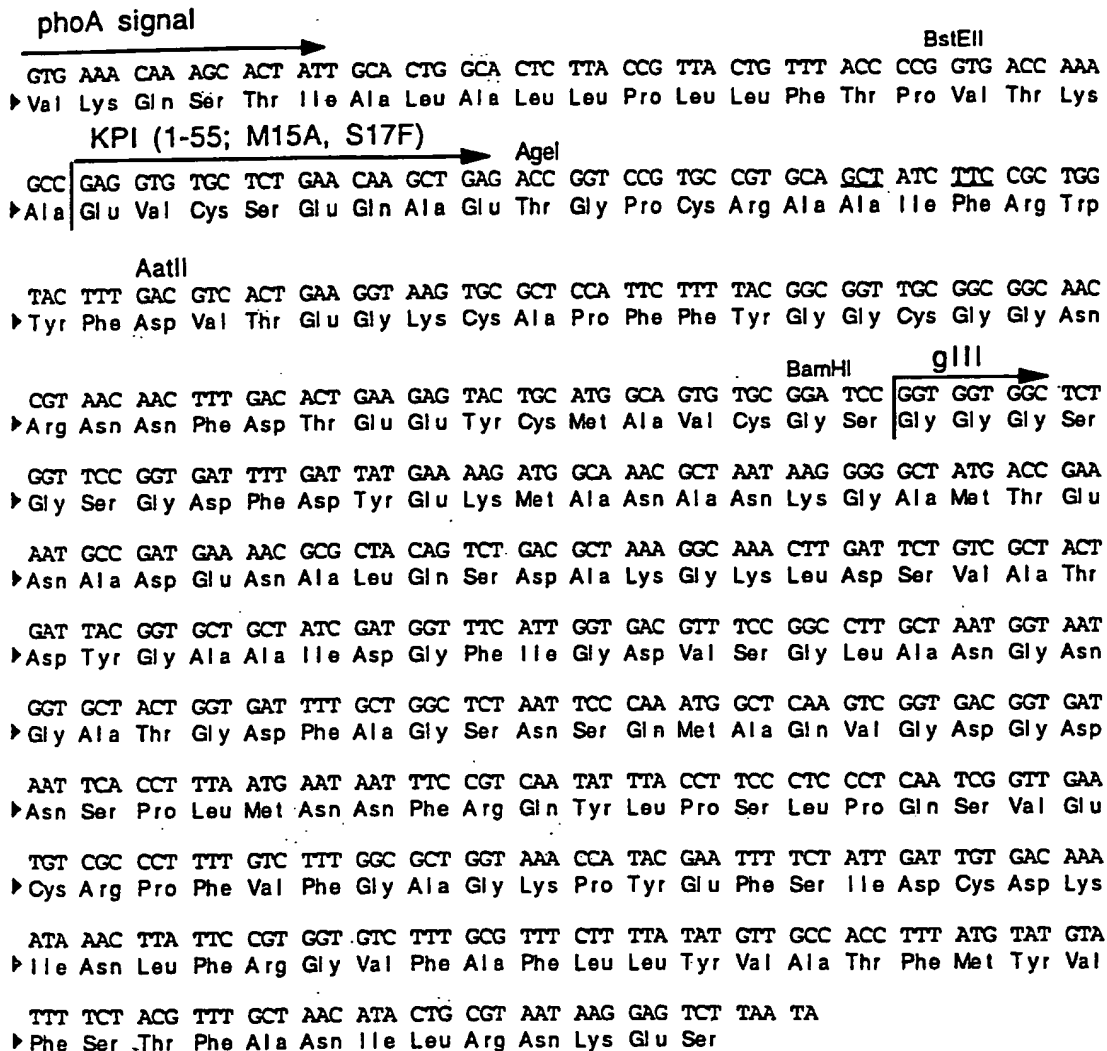
CGC CCT TTT GTC TTT GGC GCT GGT AAA CCA TAC GAA TTT TCT ATT GAT TGT GAC AAA ATA
 ▶ Arg Pro Phe Val Phe Gly Ala Gly Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile

AAC TTA TTC CGT GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT ATG TAT GTA TTT
 ▶ Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val Phe

TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT TAA TA
 ▶ Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser ...

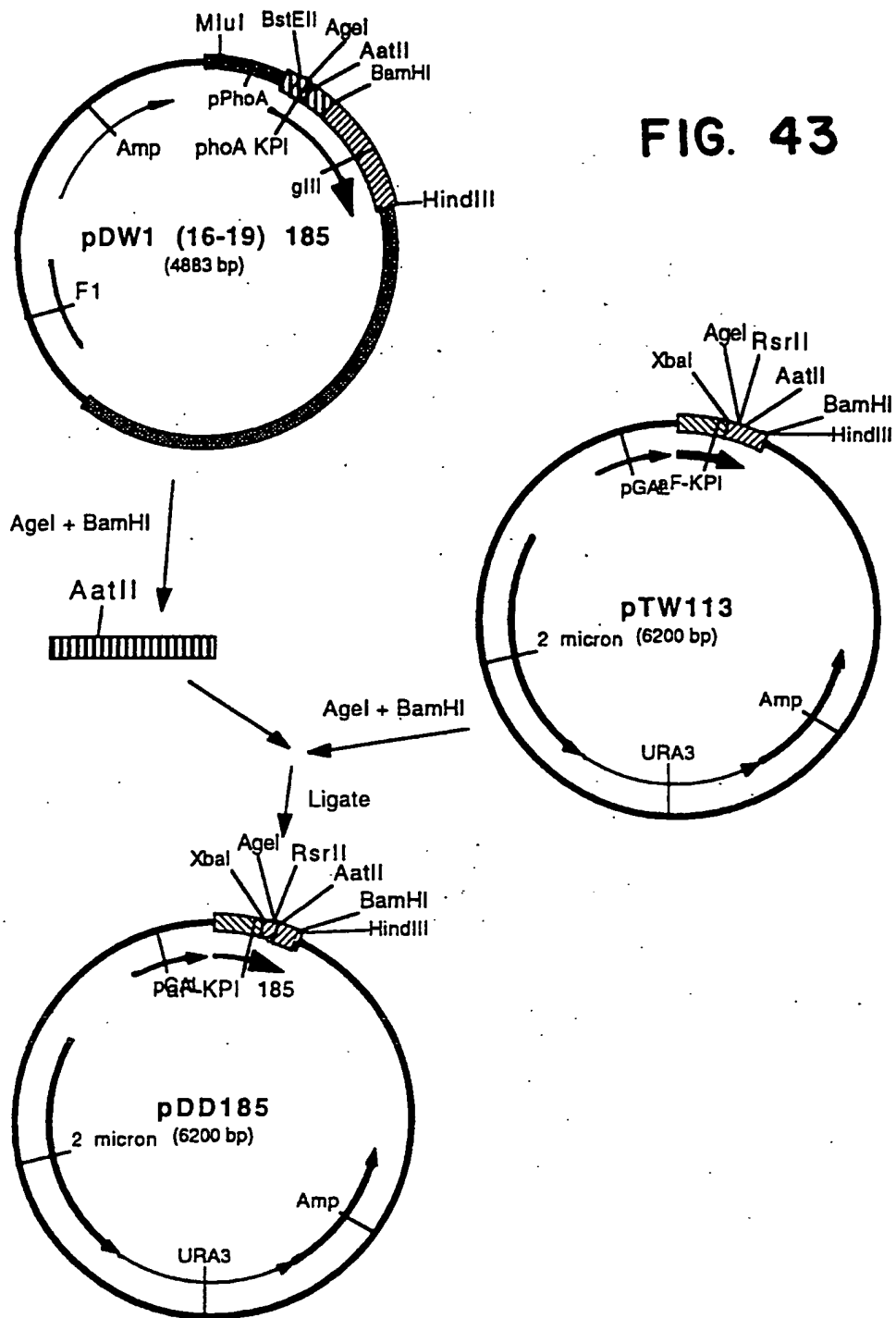
42 / 59

FIG. 42



43 / 59

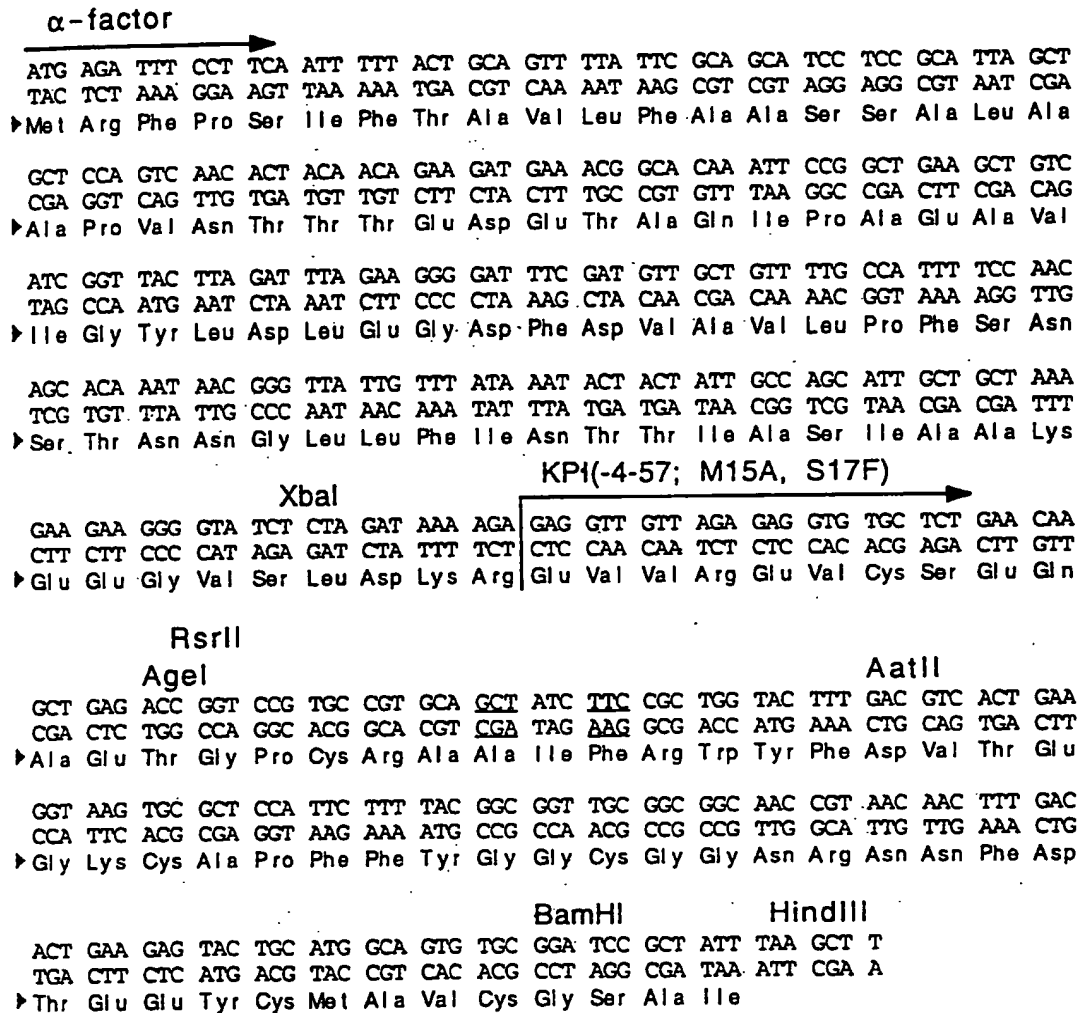
FIG. 43



44 / 59

pDD185

FIG. 44



45 / 59

Protease inhibition by KPI (-4-57) variants

K_is (nM)

| Variant | 9 | Substitution | 37 | kalikrein | X ₁₈ | X ₃₇ |
|---|---|--------------|----|-----------|-----------------|-----------------|
| TW113 KPI (-4-57) | | 15 16 17 18 | 37 | 45.00 | 3718.0 | 161.0 |
| DD185 KPI (-4-57; M15A, S17F) | | A F | | 0.39 | 150.0 | 196.0 |
| TW6165 KPI (-4-57; M15A, S17W) | | A W | | 0.65 | 206.0 | nd |
| TW6166 KPI (-4-57; M15A, S17Y) | | A Y | | 0.40 | 73.0 | nd |
| TW6175 KPI (-4-57; M15L, S17F) | | L F | | 0.50 | 35.0 | 56.0 |
| BG028 KPI (-4-57; M15L, S17Y) | | L Y | | 1.10 | 93.8 | nd |
| TW6183 KPI (-4-57; I16H, S17F) | | H F | | 1.20 | 12440.0 | 159.0 |
| TW6184 KPI (-4-57; I16H, S17Y) | | H Y | | 0.91 | 14000.0 | 214.0 |
| TW6185 KPI (-4-57; I16H, S17W) | | H W | | 1.30 | 388.0 | 473.0 |
| TW6173 KPI (-4-57; M15A, I16H) | | A H | | 1.00 | 1432.0 | nd |
| TW6174 KPI (-4-57; M15L, I16H) | | L H | | 0.90 | 2796.0 | nd |
| BG015 KPI (-4-57; M15L, S17Y, R18H) | | L Y | | 6.00 | 19.4 | 597.0 |
| BG022 KPI (-4-57; M15A, S17Y, R18H) | | A Y | | 0.64 | 14.5 | nd |
| BG029 KPI (-4-57; T9V, M15L, S17Y, R18H) | V | L Y | | 3.20 | 7.9 | nd |
| BG033 KPI (-4-57; T9V, M15A, S17Y, R18H) | V | A Y | | 0.75 | 5.8 | nd |
| DD131 KPI (-4-57; M15L, I16F, S17K) | | L F | | 7.90 | 1385.0 | 3.3 |
| DD134 KPI (-4-57; M15L, I16F, S17K, G37Y) | | L F | Y | 1.10 | 15640.0 | 0.6 |
| DD135 KPI (-4-57; M15L, I16F, S17K, G37L) | | L F | L | 1.30 | 7473.0 | 0.9 |

FIG. 45

SUBSTITUTE SHEET (RULE 26)

FIG. 46(1)

Inhibition KI (nM)

| Variant | Sequence | P ₂ kall | Plasma | X _{1/2} | X ₀ |
|--------------------|--|---------------------|--------|------------------|----------------|
| Aprotinin | RPDFCLEPPYTGPCRAKRIIRFYNAKAGLCQTFVYGGCRKRNNFKSAEDCMRTCGGA | 20.00 | 0.23 | 5000.0 | |
| Aprotinin R15, S42 | DFCLEPPYTGPCRAKRIIRFYNAKAGLCQTFVYGGCRKRNNFKSAEDCMRTCGGA | 0.91 | 0.17 | 3983.0 | |
| KPI (-4-57) | EVREVCSEQAETGPCRAMISRWFYDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 45.00 | 34.00 | 3718.0 | 161.0 |
| TW6167 | EVREVCSEQAETGPCRAMISRWFYDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 61.00 | | 3641.0 | 288.0 |
| BG031 | EVREVCSEQAETGPCRAMISRWFYDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 34.00 | | | |
| BG032 | EVREVCSEQAETGPCRAMISRWFYDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 49.00 | | 731.0 | |
| TW101 | EVREVCSEQAETGPCRAMISRWFYDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 2000.00 | 11.50 | | |
| TW6208 | EVREVCSEQAETGPCRAMISRWFYDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | | | 369.0 | |
| TW106 | EVREVCSEQAETGPCRAMISRWFYDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 560.00 | 3.70 | | |
| DD108 | EVREVCSEQAETGPCRAAISRWFYDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 1.70 | 11.20 | 1600.0 | 123.0 |
| DD109 | EVREVCSEQAETGPCRAAISRWFYDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 9.50 | | 1681.0 | 421.0 |
| DD110 | EVREVCSEQAETGPCRALISRWFYDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 2.10 | | 624.0 | 55.0 |
| DD111 | EVREVCSEQAETGPCRASISRWFYDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 5.60 | | | |
| DD112 | EVREVCSEQAETGPCRAVISRWYFDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 6.80 | | 998.0 | |
| TW6179 | EVREVCSEQAETGPCRAGISRWFYDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 78.00 | | 368.0 | |
| TW6163 | EVREVCSEQAETGPCRAMHSRWYFDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 4.70 | 103.58 | 4532.0 | 457.0 |
| TW6172 | EVREVCSEQAETGPCRAMASRWYFDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 315.00 | | | 1463.0 |
| TW6180 | EVREVCSEQAETGPCRAMFSRWYFDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 70.00 | | 885.0 | 39.0 |
| TW6181 | EVREVCSEQAETGPCRAMKSRWFYDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 150.00 | | 1514.0 | |
| BG001 | EVREVCSEQAETGPCRAMLSRWYFDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 38.00 | 10.00 | 489.0 | 204.0 |
| TW116 | EVREVCSEQAETGPCRAMISRWFYDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 145.00 | 89.00 | | 806.0 |
| DD102 | EVREVCSEQAETGPCRAMIPRWYFDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 16.00 | | 315.0 | |
| DD103 | EVREVCSEQAETGPCRAMIFRWYFDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 17.00 | | 2128.0 | 110.0 |
| DD104 | EVREVCSEQAETGPCRAMTYRWYFDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 15.00 | | 237.0 | 345.0 |
| DD105 | EVREVCSEQAETGPCRAMIWRWYFDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 18.00 | | 198.0 | 320.0 |
| TW6168 | EVREVCSEQAETGPCRAMILRWYFDVTGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 25.80 | | 3521.0 | 395.0 |

47 / 59

Inhibition KI (nM)

FIG. 46(2)

Sequence

Variant

P_{kalil} Plasma X_{lla} X_a

| | | | | | |
|--------|---|--------|-------|---------|-------|
| TW6182 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTTEEYCMVCGSAI | 36.00 | | 752.0 | |
| TW6194 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTTEEYCMVCGSAI | 70.83 | | | |
| TW6210 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTTEEYCMVCGSAI | 54.00 | | 277.0 | |
| CL008 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTTEEYCMVCGSAI | 110.20 | | 89600.0 | 133.0 |
| BG012 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTTEEYCMVCGSAI | | | 40.0 | 116.0 |
| TW6209 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTTEEYCMVCGSAI | 81.00 | 45.90 | 184.0 | 613.0 |
| TW6211 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTTEEYCMVCGSAI | 184.00 | | 402.0 | |
| DD128 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTTEEYCMVCGSAI | 44.00 | | | 37.0 |
| TW6142 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTTEEYCMVCGSAI | 18.00 | 18.00 | 7972.0 | 225.0 |
| AL301 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTTEEYCMVCGSAI | 216.00 | | 1557.0 | |
| AL302 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTTEEYCMVCGSAI | 39.00 | | | 316.0 |
| TW6147 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTTEEYCMVCGSAI | 35.00 | | 1090.0 | 179.0 |
| TW6138 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCKGNRNNFDTTEEYCMVCGSAI | 18.00 | | 921.0 | 309.0 |
| TW6154 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCLGNRNNFDTTEEYCMVCGSAI | 11.00 | | 915.0 | 39.0 |
| TW6155 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCMGNRNNFDTTEEYCMVCGSAI | 11.00 | | | 27.0 |
| TW6140 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTTEEYCMVCGSAI | 35.00 | | 475.0 | |
| TW6158 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCPGNRNNFDTTEEYCMVCGSAI | | | | |
| TW6141 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCQGNRNNFDTTEEYCMVCGSAI | 42.00 | | | |
| TW118 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTTEEYCMVCGSAI | 6.00 | 24.00 | 13009.0 | 68.0 |
| DD100 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTTEEYCMVCGSAI | 15.00 | | | |
| TW6157 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCSGNRNNFDTTEEYCMVCGSAI | 40.00 | | 511.0 | 168.0 |
| TW6158 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCTGNRNNFDTTEEYCMVCGSAI | 29.00 | | | |
| TW6159 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTTEEYCMVCGSAI | 17.00 | | | 64.0 |
| TW6161 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCYGNRNNFDTTEEYCMVCGSAI | 7.50 | 18.00 | 1507.0 | 8.7 |
| DD101 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCDGNRNNFDTTEEYCMVCGSAI | 64.00 | | 924.0 | |
| TW6151 | EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCEGNRNNFDTTEEYCMVCGSAI | 163.00 | | 1162.0 | 954.0 |

Inhibition KI (nM)

FIG. 46(3)

| Variant | Sequence | P _{kalii} | Plasma | X _{la} | X _a |
|---------|---|--------------------|--------|-----------------|----------------|
| TW6139 | EVVREVCSEQAETGPCRAMISRWFYFDVTEGKCAPFFYGGCHGRNNFDTTEYCMVAVCGSAI | 19.00 | 22.80 | 152.0 | 78.0 |
| TW6153 | EVVREVCSEQAETGPCRAMISRWFYFDVTEGKCAPFFYGGCIGNNNFDTTEYCMVAVCGSAI | 11.20 | 21.30 | 65.0 | 38.0 |
| TW122 | EVVREVCSEQAETGPCRAMISRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 32.00 | 27.00 | | 581.0 |
| TW6178 | EVVREVCSEQAETGPCRAMISRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 16.00 | | 444.0 | |
| TW6148 | EVVREVCSEQAETGPCRAMISRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 40.00 | | | |
| TW124 | EVVREVCSEQAETGPCRAMISRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 64.00 | 48.00 | | |
| TW6149 | EVVREVCSEQAETGPCRAMISRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 54.00 | | | |
| TW6173 | EVVREVCSEQAETGPCRAAHSRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 1.00 | 7.24 | 1432.0 | |
| TW6174 | EVVREVCSEQAETGPCRALHSRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 0.90 | 6.89 | 2796.0 | |
| BG002 | EVVREVCSEQAETGPCRALLSRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 0.98 | 19.00 | 403.0 | 60.0 |
| DD129 | EVVREVCSEQAETGPCRALFSRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 3.60 | | 1864.0 | 8.0 |
| DD185 | EVVREVCSEQAETGPCRAALIRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 0.39 | 8.71 | 150.0 | 198.0 |
| TW6165 | EVVREVCSEQAETGPCRAALIRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 0.65 | 16.40 | 206.0 | |
| TW6186 | EVVREVCSEQAETGPCRAALIRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 0.40 | 10.10 | 73.0 | |
| BG028 | EVVREVCSEQAETGPCRALIRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 1.10 | 12.10 | 93.8 | |
| TW6169 | EVVREVCSEQAETGPCRALILRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 1.20 | | 619.0 | 111.0 |
| DD113 | EVVREVCSEQAETGPCRALIPRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 0.85 | 12.80 | 293.0 | 74.0 |
| TW6175 | EVVREVCSEQAETGPCRALIPRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 0.50 | 7.46 | 35.0 | 56.0 |
| TW6201 | EVVREVCSEQAETGPCRAGIRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 34.60 | | 419.0 | |
| TW6202 | EVVREVCSEQAETGPCRAGIRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 128.50 | | 1237.0 | |
| TW6203 | EVVREVCSEQAETGPCRAGIRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 31.20 | | 5045.0 | |
| TW6204 | EVVREVCSEQAETGPCRAAISRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | | | 147.0 | 87.0 |
| TW6205 | EVVREVCSEQAETGPCRALISRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | | | 195.0 | 29.0 |
| DD114 | EVVREVCSEQAETGPCRAAISRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 0.70 | 7.77 | 224.0 | |
| TW6190 | EVVREVCSEQAETGPCRAAISRWFYFDVTEGKCAPFFYGGCYGNRNNFDTTEYCMVAVCGSAI | 0.83 | 52.20 | 589.0 | 1396.0 |
| TW6183 | EVVREVCSEQAETGPCRAMHFRWFYFDVTEGKCAPFFYGGCGNRNNFDTTEYCMVAVCGSAI | 1.20 | 11.68 | 12440.0 | 159.0 |

FIG. 46(4)

| Variant | Sequence | P.kall | Plasmin | Xlla | Xa |
|---------|--|--------|---------|---------|-------|
| TW6184 | EVVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 0.91 | 11.96 | 14000.0 | 214.0 |
| TW6185 | EVVREVCSEQAETGPCRAMHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 1.30 | 18.60 | 388.0 | 473.0 |
| BG003 | EVVREVCSEQAETGPCRAMLHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 38.00 | | 467.0 | |
| TW6186 | EVVREVCSEQAETGPCRAMHSRWYFDVTEGKCAPFFYGGCYGNRNNFDTEEYCMVCGSAI | 0.48 | 8.86 | 186.0 | 11.0 |
| TW6187 | EVVREVCSEQAETGPCRAMIFRWYFDVTEGKCAPFFYGGCYGNRNNFDTEEYCMVCGSAI | 3.80 | 15.40 | 92.0 | 15.0 |
| TW6188 | EVVREVCSEQAETGPCRAMIWRWYFDVTEGKCAPFFYGGCYGNRNNFDTEEYCMVCGSAI | 4.00 | | 419.0 | 24.0 |
| TW6189 | EVVREVCSEQAETGPCRAMIWRWYFDVTEGKCAPFFYGGCYGNRNNFDTEEYCMVCGSAI | 4.00 | | | 34.0 |
| TW6170 | EVVREVCSEQAETGPCRALILRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 2.50 | | | 452.0 |
| DD115 | EVVREVCSEQAETGPCRGYITRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | | | 213.0 | 299.0 |
| DD170 | EVVREVCSEQAETGPCRALHNRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 0.99 | 18.00 | 550.0 | |
| TW6176 | EVVREVCSEQAETGPCRAAHFRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 3.50 | 118.00 | 56.0 | |
| TW6177 | EVVREVCSEQAETGPCRALHFRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 7.20 | 32.70 | 245.0 | 156.0 |
| BG006 | EVVREVCSEQAETGPCRAALFRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 0.30 | 12.10 | 80.0 | |
| DD130 | EVVREVCSEQAETGPCRALFTRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 5.50 | | | 9.5 |
| DD131 | EVVREVCSEQAETGPCRALFKRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 7.90 | 2.00 | 1385.0 | 3.3 |
| DD132 | EVVREVCSEQAETGPCRAFFKRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 112.00 | | | 16.8 |
| DD120 | EVVREVCSEQAETGPCRAAFSAWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 8.30 | | | 11.0 |
| DD121 | EVVREVCSEQAETGPCRALLSAWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 19.00 | | | 21.0 |
| BG014 | EVVREVCSEQAETGPCRALIHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 9.20 | 18.70 | 18.0 | |
| DD122 | EVVREVCSEQAETGPCRALIFAWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 15.00 | | | 46.0 |
| BG015 | EVVREVCSEQAETGPCRALIYHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 6.00 | 12.20 | 19.4 | 597.0 |
| BG020 | EVVREVCSEQAETGPCRAAIHKWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 1.70 | | 106.0 | |
| BG022 | EVVREVCSEQAETGPCRAAIYHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 0.84 | 7.26 | 14.5 | |
| BG023 | EVVREVCSEQAETGPCRALIQHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 23.00 | | 262.0 | |
| BG024 | EVVREVCSEQAETGPCRALIKWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 4.10 | 7.47 | 36.7 | |
| BG027 | EVVREVCSEQAETGPCRAAIQHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMVCGSAI | 5.80 | | 144.0 | |

50 / 59

FIG. 46(5)

| Variant | Sequence | Inhibition Ki (nM) | | | |
|---------|---|--------------------|---------|-----------------|----------------|
| | | P _{kal} | Plasmid | X ₁₀ | X ₂ |
| DD116 | EVREVCSEQAETGPCRAAIFRWYFDVTEGKCAPFFYGGCGNRNRFDTTEYCMVCGSAI | 0.14 | | 583.0 | 84.0 |
| TW6191 | EVREVCSEQAETGPCRAAIFRWYFDVTEGKCAPFFYGGCGNRNRFDTTEYCMVCGSAI | 0.26 | | 664.0 | 20.0 |
| DD117 | EVREVCSEQAETGPCRALIPRWYFDVTEGKCAPFFYGGCGNRNRFDTTEYCMVCGSAI | 0.11 | | 1034.0 | 99.0 |
| BG029 | EVREVCSEQAETGPCRALIYHWYFDVTEGKCAPFFYGGCGNRNRFDTTEYCMVCGSAI | 3.20 | | 7.9 | |
| BG030 | EVREVCSEQAETGPCRALIYHWYFDVTEGKCAPFFYGGCGNRNRFDTTEYCMVCGSAI | 4.60 | | 26.1 | |
| BG033 | EVREVCSEQAETGPCRAAIIHWYFDVTEGKCAPFFYGGCGNRNRFDTTEYCMVCGSAI | 0.75 | | 5.8 | |
| BG034 | EVREVCSEQAETGPCRAAIIHWYFDVTEGKCAPFFYGGCGNRNRFDTTEYCMVCGSAI | 0.47 | | 18.5 | |
| BG040 | EVREVCSEQAETGPCRALIYHWYFDVTEGKCAPFFYGGCGNRNRFDTTEYCMVCGSAI | 3.40 | | 8.8 | |
| BG016 | EVREVCSEQAETGPCRGAIQHWYFDVTEGKCAPFFYGGCGNRNRFDTTEYCMVCGSAI | 160.00 | | 178.0 | |
| BG017 | EVREVCSEQAETGPCRGAIIRHWYFDVTEGKCAPFFYGGCGNRNRFDTTEYCMVCGSAI | 180.00 | | 200.0 | |
| BG021 | EVREVCSEQAETGPCRSIRHWYFDVTEGKCAPFFYGGCGNRNRFDTTEYCMVCGSAI | 340.00 | | 224.0 | |
| BG025 | EVREVCSEQAETGPCRGIIYHWYFDVTEGKCAPFFYGGCGNRNRFDTTEYCMVCGSAI | 65.00 | | 16.2 | |
| BG026 | EVREVCSEQAETGPCRGAIYHWYFDVTEGKCAPFFYGGCGNRNRFDTTEYCMVCGSAI | 50.00 | | 34.9 | |
| DD118 | EVREVCSEQAETGPCRALHNRWYFDVTEGKCAPFFYGGCGNRNRFDTTEYCMVCGSAI | 0.53 | | | |
| DD134 | EVREVCSEQAETGPCRALFKRWYFDVTEGKCAPFFYGGCGNRNRFDTTEYCMVCGSAI | 1.10 | 1.05 | 15840.0 | 0.6 |
| DD135 | EVREVCSEQAETGPCRALFKRWYFDVTEGKCAPFFYGGCGNRNRFDTTEYCMVCGSAI | 1.30 | | 7473.0 | 0.9 |
| DD136 | EVREVCSEQAETGPCRALFKRWYFDVTEGKCAPFFYGGCGNRNRFDTTEYCMVCGSAI | 1.10 | | | 1.8 |

SUBSTITUTE SHEET (RULE 26)

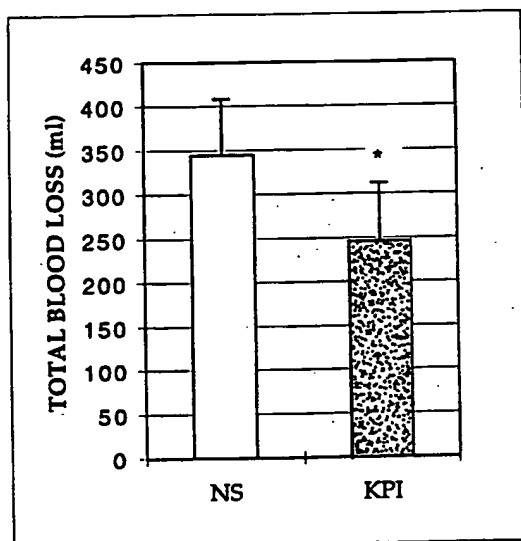
51 / 59

FIG. 47

VOLUMES

| | |
|-----|--------|
| NS | 344.25 |
| KPI | 245.75 |

| | KPI | NS |
|-------|-------------|-------------|
| | 298 | 366 |
| | 266 | 342 |
| | 354 | 294 |
| | 258 | 385 |
| | 168 | 288 |
| | 266 | 469 |
| | 172 | 338 |
| | 184 | 272 |
| MEAN | 245.75 | 344.25 |
| STDEV | 66.2414415 | 63.97488346 |
| TTEST | 0.009094999 | |



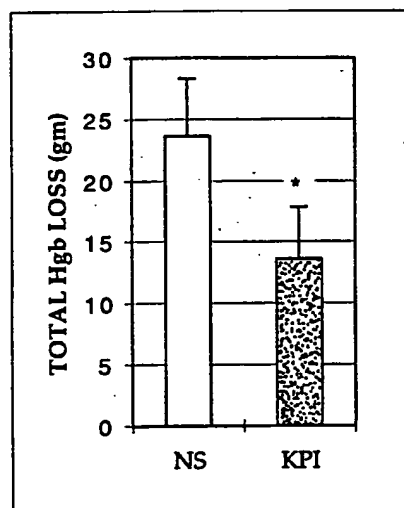
52 / 59

FIG. 48

HEMOGLOBIN

| | |
|-----|-------|
| NS | 23.61 |
| KPI | 13.59 |

| | KPI | NS |
|-------|----------|----------|
| | 16.58 | 24.95 |
| | 15.19 | 24.87 |
| | 20.21 | 20.46 |
| | 8.99 | 27.59 |
| | 14.63 | 18.23 |
| | 15.31 | 31.59 |
| | 7.7 | 23.26 |
| | 10.14 | 17.96 |
| MEAN | 13.59375 | 23.61375 |
| STDEV | 4.261438 | 4.68761 |
| TTEST | | 0.000536 |



53 / 59

FIG. 49

PaO₂

| | Baseline PaO ₂ | | End CPB | | Obs 60 min | | Obs 180 min | |
|-------|---------------------------|----------|-----------|----------|------------|----------|-------------|---------|
| | KPI | NS | KPI | NS | KPI | NS | KPI | NS |
| MEAN | 652.2 | 670.9 | 495.7 | 60.5 | 483.7 | 441.3 | 391.3 | |
| STDEV | 654 | 559.2 | 444.6 | 132.2 | 330.1 | 448.7 | 264.1 | 484.6 |
| TTEST | 596.2 | 622.9 | 170.2 | 93.8 | 415.4 | 85.1 | 416.5 | 81.3 |
| | 606.2 | 689.2 | 264.2 | 333.9 | 430.2 | 529.6 | 361.9 | 333.2 |
| | 633.1 | 665.1 | 567.2 | 341.7 | 613 | 568.3 | 90.8 | 546.6 |
| | 646.6 | 527 | 507.4 | 226.9 | 564.3 | 438.1 | 518.2 | 485.3 |
| | 563.2 | 461.7 | 547.1 | 89.1 | 501 | 42.6 | 494.2 | 45.6 |
| | 659.9 | 508 | 416.6 | 59.7 | 504.5 | 405.8 | 452 | 383.7 |
| | 626.425 | 588 | 426.625 | 167.225 | 480.275 | 369.938 | 371.1 | 344 |
| | 34.46923 | 85.50556 | 140.4741 | 117.9931 | 88.61879 | 196.5235 | 150.2774 | 186.227 |
| | p= 0.268 | | p= 0.0014 | | p= 0.17915 | | p= 0.76 | |

N.S.

N.S.

54 / 59

Summary of Data

FIG. 50

| Total Volumes | | Serial Chest tube Hb/g | | | | | |
|----------------------|------|------------------------|----------|-----------|------------|-----|--|
| Chest tube Sacrifice | | 0-30min | 30-60min | 60-120min | 120-180min | | |
| KPI-1 | 185 | 113 | 3.7 | 4.3 | 8.6 | 6.2 | |
| KPI-2 | 198 | 68 | 4.3 | 6.4 | 6.7 | 5.7 | |
| KPI-3 | 142 | 212 | 4.1 | 4.4 | 7 | 7.1 | |
| KPI-4 | 190 | 68 | 2.8 | 4 | 4.4 | 1.9 | |
| KPI-5 | 96 | 72 | 6.3 | 6.5 | 7 | 6.7 | |
| KPI-6 | 188 | 78 | 4.1 | 6.1 | 5.6 | 6.3 | |
| KPI-7 | 134 | 38 | 3.1 | 4.6 | 5.4 | 4.4 | |
| KPI-8 | 158 | 26 | 6.9 | 5.8 | 5.4 | 4.2 | |
| MEAN | 4.41 | 5.26 | 6.26 | 5.3 | | | |
| STDEV | 1.45 | 1.04 | 1.32 | 1.72 | | | |

| Total volume loss | | Total Hgb Loss | |
|-------------------|--------|----------------|--|
| KPI-1 | 298 | 16.58 | |
| KPI-2 | 266 | 15.19 | |
| KPI-3 | 354 | 20.21 | |
| KPI-4 | 258 | 8.99 | |
| KPI-5 | 168 | 14.63 | |
| KPI-6 | 266 | 15.31 | |
| KPI-7 | 172 | 7.7 | |
| KPI-8 | 184 | 10.14 | |
| MEAN | 245.75 | 13.59 | |
| STDEV | 66.24 | 4.26 | |

| Total Volumes | | Serial Chest tube Hb/g | | | | | |
|----------------------|------|------------------------|----------|-----------|------------|-----|--|
| Chest tube Sacrifice | | 0-30min | 30-60min | 60-120min | 120-180min | | |
| NS-1A | 274 | 92 | 7.7 | 8.6 | 6.1 | 5.4 | |
| NS-2 | 236 | 106 | 7.2 | 7.4 | 7.6 | 7.1 | |
| NS-3 | 252 | 42 | 5.4 | 7.5 | 7.5 | 6.5 | |
| NS-4 | 303 | 82 | 8.4 | 7.2 | 7.1 | 6.3 | |
| NS-5 | 140 | 148 | 7.5 | 7.2 | 5.2 | 5.6 | |
| NS-6 | 261 | 208 | 4 | 7 | 7.3 | 7.4 | |
| NS-7 | 218 | 120 | 7.5 | 7.7 | 5.8 | 4.2 | |
| NS-8 | 206 | 66 | 7.4 | 8.2 | 6 | 5.3 | |
| MEAN | 6.89 | 7.6 | 6.58 | 6.1 | | | |
| STDEV | 1.44 | 1.04 | 0.91 | 0.85 | | | |

| Total volume loss | | Total Hgb Loss | |
|-------------------|--------|----------------|--|
| NS-1A | 366 | 24.95 | |
| NS-2 | 342 | 24.87 | |
| NS-3 | 294 | 20.46 | |
| NS-4 | 385 | 27.59 | |
| NS-5 | 288 | 18.23 | |
| NS-6 | 469 | 31.59 | |
| NS-7 | 338 | 23.26 | |
| NS-8 | 272 | 17.96 | |
| MEAN | 344.25 | 23.61 | |
| STDEV | 63.97 | 4.69 | |

*p = 0.0004

*p = 0.0002

NS

NS

*p = 0.0005

*p = 0.0009

*p = 0.004 *p = 0.0002 NS NS

*p = 0.009 *p = 0.0005

55 / 59

pTW 6166

FIG. 51

 α -factor

ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
 TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
 ▶ Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

 GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
 CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
 ▶ Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

 ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
 TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
 ▶ Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

 AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
 TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
 ▶ Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

XbaI

KPI(-4-57; M15A, S17Y)

GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
 CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
 ▶ Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln

RsrII

AgeI

AatII

GCT GAG ACC GGT CCG TGC CGT GCA GCT ATC TAC CGC TGG TAC TTT GAC GTC ACT GAA
 CGA CTC TGG CCA GGC ACG GCA CGT CGA TAG ATG GCG ACC ATG AAA CTG CAG TGA CTT
 ▶ Ala Glu Thr Gly Pro Cys Arg Ala Ala Ile Tyr Arg Trp Tyr Phe Asp Val Thr Glu

 GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
 CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
 ▶ Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

BamHI

HindIII

ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
 TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
 ▶ Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

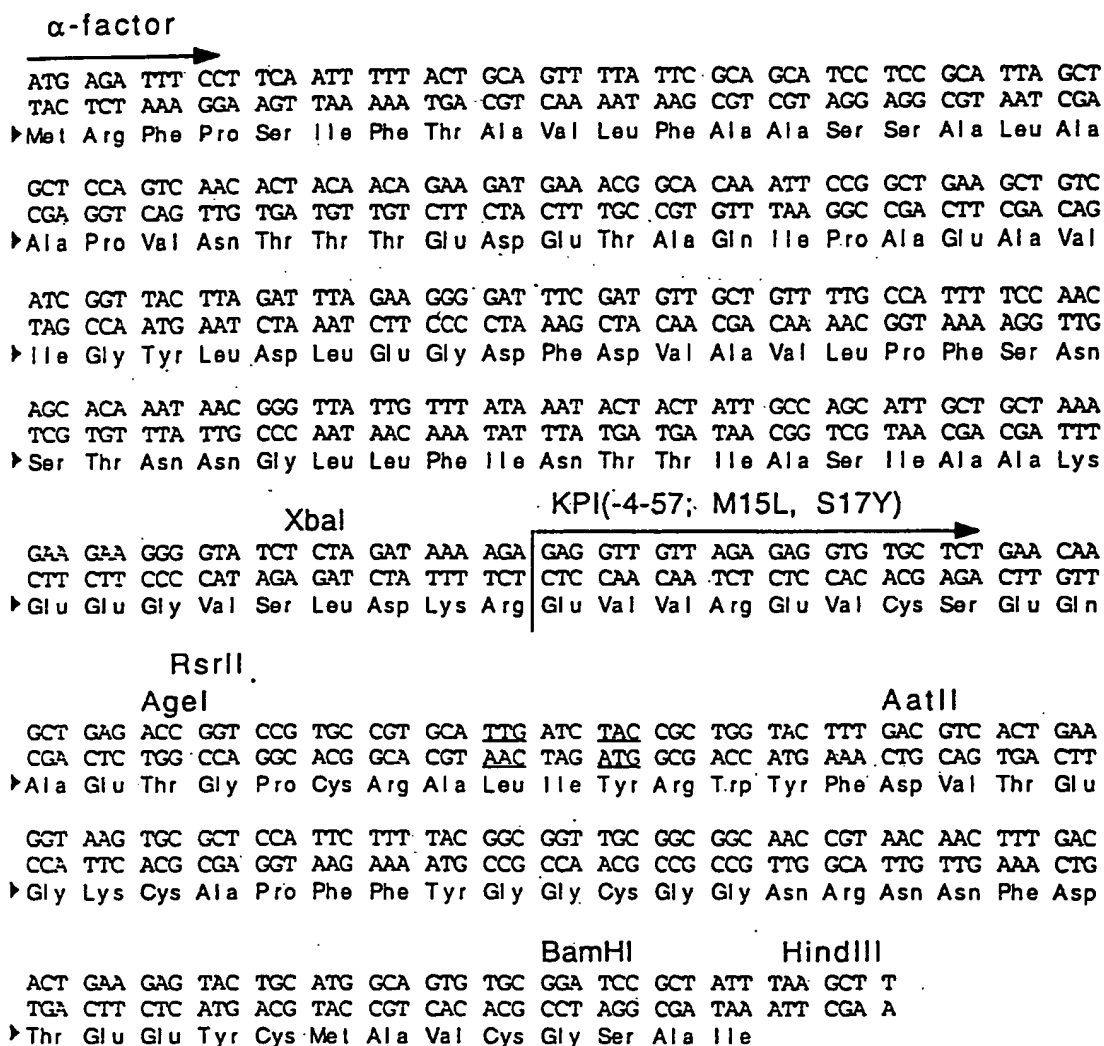
56 / 59

FIG. 52

| α -factor | | | | | | | | | | | | | | | | | | | | | | | |
|------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|---------|-----|-----|-----|-----|-----|-----|--|--|--|--|--|
| ATG | AGA | TTT | CCT | TCA | ATT | TTT | ACT | GCA | GTT | TTA | TTC | GCA | GCA | TCC | TCC | GCA | TTA | GCT | | | | | |
| TAC | TCT | AAA | GGA | AGT | TAA | AAA | TGA | CGT | CAA | AAT | AAG | CGT | CGT | AGG | AGG | CGT | AAT | CGA | | | | | |
| Met | Arg | Phe | Pro | Ser | Ile | Phe | Thr | Ala | Val | Leu | Phe | Ala | Ala | Ser | Ser | Ala | Leu | Ala | | | | | |
| GCT | CCA | GTC | AAC | ACT | ACA | ACA | GAA | GAT | GAA | ACG | GCA | CAA | ATT | CCG | GCT | GAA | GCT | GTC | | | | | |
| CGA | GGT | CAG | TTG | TGA | TGT | TGT | CTT | CTA | CTT | TGC | CGT | GTT | TAA | GGC | CGA | CTT | CGA | CAG | | | | | |
| Ala | Pro | Val | Asn | Thr | Thr | Thr | Glu | Asp | Glu | Thr | Ala | Gln | Ile | Pro | Ala | Glu | Ala | Val | | | | | |
| ATC | GGT | TAC | TTA | GAT | TTA | GAA | GGG | GAT | TTC | GAT | GTT | GCT | GTT | TTG | CCA | TTT | TCC | AAC | | | | | |
| TAG | CCA | ATG | AAT | CTA | AAT | CTT | CCC | CTA | AAG | CTA | CAA | CGA | CAA | AAC | GGT | AAA | AGG | TTG | | | | | |
| Ile | Gly | Tyr | Leu | Asp | Leu | Glu | Gly | Asp | Phe | Asp | Val | Ala | Val | Leu | Pro | Phe | Ser | Asn | | | | | |
| AGC | ACA | AAT | AAC | GGG | TTA | TTG | TTT | ATA | AAT | ACT | ACT | ATT | GCC | AGC | ATT | GCT | GCT | AAA | | | | | |
| TCG | TGT | TTA | TTG | CCC | AAT | AAC | AAA | TAT | TTA | TGA | TGA | TAA | CGG | TCG | TAA | CGA | CGA | TTT | | | | | |
| Ser | Thr | Asn | Asn | Gly | Leu | Leu | Phe | Ile | Asn | Thr | Thr | Ile | Ala | Ser | Ile | Ala | Ala | Lys | | | | | |
| KPI(-4-57; M15L, S17F) | | | | | | | | | | | | | | | | | | | | | | | |
| XbaI | | | | | | | | | | | | | | | | | | | | | | | |
| GAA | GAA | GGG | GTA | TCT | CTA | GAT | AAA | AGA | GAG | GTT | GTT | AGA | GAG | GTG | TGC | TCT | GAA | CAA | | | | | |
| CTT | CTT | CCC | CAT | AGA | GAT | CTA | TTT | TCT | CTC | CAA | CAA | TCT | CTC | CAC | ACG | AGA | CTT | GTT | | | | | |
| Glu | Glu | Gly | Val | Ser | Leu | Asp | Lys | Arg | Glu | Val | Val | Arg | Glu | Val | Cys | Ser | Glu | Gln | | | | | |
| RsrII | | | | | | | | | | | | | | | | | | | | | | | |
| AgeI | | | | | | | | | | | | AatII | | | | | | | | | | | |
| GCT | GAG | ACC | GGT | CCG | TGC | CGT | GCA | TTG | ATC | TTT | CGC | TGG | TAC | TTT | GAC | GTC | ACT | GAA | | | | | |
| CGA | CTC | TGG | CCA | GGC | ACG | GCA | CGT | AAC | TAG | AAG | GCG | ACC | ATG | AAA | CTG | CAG | TGA | CTT | | | | | |
| Ala | Glu | Thr | Gly | Pro | Cys | Arg | Ala | Leu | Ile | Phe | Arg | Trp | Tyr | Phe | Asp | Val | Thr | Glu | | | | | |
| GGT | AAG | TGC | GCT | CCA | TTC | TTT | TAC | GGC | GGT | TGC | GGC | GGC | AAC | CGT | AAC | AAC | TTT | GAC | | | | | |
| CCA | TTC | ACG | CGA | GGT | AAG | AAA | ATG | CCG | CCA | ACG | CCG | CCG | TTG | GCA | TTG | TTG | AAA | CTG | | | | | |
| Gly | Lys | Cys | Ala | Pro | Phe | Phe | Tyr | Gly | Gly | Cys | Gly | Gly | Asn | Arg | Asn | Asn | Phe | Asp | | | | | |
| BamHI | | | | | | | | | | | | HindIII | | | | | | | | | | | |
| ACT | GAA | GAG | TAC | TGC | ATG | GCA | GTG | TGC | GGA | TCC | GCT | ATT | TAA | GCT | T | | | | | | | | |
| TGA | CTT | CTC | ATG | ACG | TAC | CGT | CAC | ACG | CCT | AGG | CGA | TAA | ATT | CGA | A | | | | | | | | |
| Thr | Glu | Glu | Tyr | Cys | Met | Ala | Val | Cys | Gly | Ser | Ala | Ile | | | | | | | | | | | |

57 / 59

FIG. 53



58 / 59

FIG. 54(1)

| PROTEIN | SEQUENCE | K _i kallikrein | K _i Factor XIIa | K _i Plasmin |
|-------------|---|------------------------------|----------------------------------|---------------------------|
| Aprotinin | RPDFCLEPPYTGPCRAIIRVFNKAGLCQIFVYGGCRAXNNFKSADCHRTCCGA | 22.6 | 5000 | 0.33 |
| KPI (-4-57) | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | 45.0 | 3718.0 | 34.00 |
| TH101 | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | >5000 | nd | 12.30 |
| TH106 | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | 449.0 | nd | 2.98 |
| TH116 | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | 116.00 | nd | 70.90 |
| TH105 | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | >5000 | nd | 1.45 |
| TH117 | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | >5000 | nd | 19.90 |
| TH115 | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | 671.0 | nd | 2.24 |
| TH102 | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | >5000 | nd | 1.27 |
| CL005 | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | >5000 | >5000 | >5000 |
| TH6172 | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | 315.0 | nd | 1555.0 |
| TH6207 | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | 54.0 | 635.0 | 44.10 |
| CL0062 | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | 110.2 | 89600 | 31.10 |
| DD108 | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | 1.7 | 1600.0 | 11.20 |
| DD110 | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | 2.1 | 624.0 | 11.000 |
| DD111 | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | 5.6 | nd | nd |
| DD112 | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | 6.8 | 998.0 | nd |
| DD102 | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | 16.0 | 315.0 | nd |
| DD103 | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | 17.0 | 2128.0 | nd |
| DD104 | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | 15.0 | 237.0 | nd |
| DD105 | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | 18.0 | 198.0 | nd |
| TH6166 | EVVREVCSEQAETGPCRAHISRNYFDVTEGKCAPFFYGGCGGNRRNFDTEEYCHAVCGSAI | 0.4 | 73.0 | 10.10 |

59 / 59

FIG. 54(2)

| | | | | |
|--------|---|-----|-------|-------|
| TH6165 | EVVREVCSEQAETGPCRAAIWRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI | .65 | 206.0 | 16.4 |
| BG028 | EVVREVCSEQAETGPCRALIYRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI | 1.1 | 93.8 | 12.10 |
| TH6175 | EVVREVCSEQAETGPCRALIFRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI | 0.5 | 35.0 | 7.46 |
| TH6238 | EVVREVCSEQAETGPCRAIHHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI | 2.5 | 40.0 | nd |
| TH6245 | EVVREVCSEQAETGPCRAIHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI | 9.9 | 76 | nd |
| TH6247 | EVVREVCSEQAETGPCRAIHHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI | 4.6 | 38 | nd |

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | | | |
|--|--|-----------|--|--|
| (51) International Patent Classification ⁶ : C12N 15/15, C07K 14/81, C12N 1/21 A61K 38/57, C12N 1/19, 15/62 | | A3 | (11) International Publication Number: WO 96/35788 (43) International Publication Date: 14 November 1996 (14.11.96) | |
| (21) International Application Number: PCT/US96/06384 (22) International Filing Date: 8 May 1996 (08.05.96) (30) Priority Data: 08/436,555 8 May 1995 (08.05.95) US 08/643,731 6 May 1996 (06.05.96) US (60) Parent Applications or Grants (63) Related by Continuation US 08/436,555 (CON) Filed on 8 May 1995 (08.05.95) US 08/643,731 (CON) Filed on 6 May 1996 (06.05.96) (71) Applicant (for all designated States except US): SCIOS, INC. [US/US]; 2450 Bayshore Parkway, Mountain View, CA 94043 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): WHITE, Tyler, R. [US/US]; 41600 Marigold Drive, Fremont, CA 94539 (US). DAMM, Deborah [US/US]; 711 Temescal Way, Redwood City, CA 94062 (US). LESIKAR, David, D. [US/US]; 2291 South Court, Palo Alto, CA 94301-4134 | | | (74) Agents: PELTO, Don, J. et al.; Foley & Lardner, Suite 500, 3000 K Street, N.W., Washington, DC 20007-5109 (US). (81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 12 December 1996 (12.12.96) | |
| (54) Title: KUNITZ TYPE PROTEASE INHIBITORS | | | | |
| (57) Abstract <p>Analogues of the Kunitz Protease Inhibitor (KPI) domain of amyloid precursor protein bind to and inhibit activity of serine proteases, including kallikrein, plasmin and coagulation factors such as factors VIIa, IXa, Xa, XIa and XIIa. Pharmaceutical compositions containing the KPI analogues, along with methods for using such compositions, are useful for ameliorating and treating clinical conditions associated with increased serine protease activity, such as blood loss related to cardiopulmonary bypass surgery. Nucleic acid sequences encoding these analogues and systems for expression of the peptides of the invention are provided.</p> | | | | |

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|----|--------------------------|----|--|----|--------------------------|
| AM | Armenia | GB | United Kingdom | MW | Malawi |
| AT | Austria | GE | Georgia | MX | Mexico |
| AU | Australia | GN | Guinea | NE | Niger |
| BB | Barbados | GR | Greece | NL | Netherlands |
| BE | Belgium | HU | Hungary | NO | Norway |
| BF | Burkina Faso | IE | Ireland | NZ | New Zealand |
| BG | Bulgaria | IT | Italy | PL | Poland |
| BJ | Benin | JP | Japan | PT | Portugal |
| BR | Brazil | KE | Kenya | RO | Romania |
| BY | Belarus | KG | Kyrgyzstan | RU | Russian Federation |
| CA | Canada | KP | Democratic People's Republic of Korea | SD | Sudan |
| CF | Central African Republic | KR | Republic of Korea | SE | Sweden |
| CG | Congo | KZ | Kazakhstan | SG | Singapore |
| CH | Switzerland | LI | Liechtenstein | SI | Slovenia |
| CI | Côte d'Ivoire | LK | Sri Lanka | SK | Slovakia |
| CM | Cameroon | LR | Liberia | SN | Senegal |
| CN | China | LT | Lithuania | SZ | Swaziland |
| CS | Czechoslovakia | LU | Luxembourg | TD | Chad |
| CZ | Czech Republic | LV | Latvia | TG | Togo |
| DE | Germany | MC | Monaco | TJ | Tajikistan |
| DK | Denmark | MD | Republic of Moldova | TT | Trinidad and Tobago |
| EE | Estonia | MG | Madagascar | UA | Ukraine |
| ES | Spain | ML | Mali | UG | Uganda |
| FI | Finland | MN | Mongolia | US | United States of America |
| FR | France | MR | Mauritania | UZ | Uzbekistan |
| GA | Gabon | | | VN | Viet Nam |

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/06384

| A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/15 C07K14/81 C12N1/21 A61K38/57 C12N1/19 C12N15/62 | | |
|---|---|--|
| According to International Patent Classification (IPC) or to both national classification and IPC | | |
| B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C07K | | |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched | | |
| Electronic data base consulted during the international search (name of data base and, where practical, search terms used) | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | |
| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| Y | BIOCHEMISTRY 29 (43). 1990. 10018-10022. CODEN: BICHAW ISSN: 0006-2960, XP002015891 HYNES T R ET AL: "X-RAY CRYSTAL STRUCTURE OF THE PROTEASE INHIBITOR DOMAIN OF ALZHEIMER 'S AMYLOID BETA-PROTEIN PRECURSOR." see the whole document --- | 1-133 |
| Y | JOURNAL OF MOLECULAR BIOLOGY, vol. 230, 1993, pages 919-933, XP000608106 J. PERONA ET AL: "Crystal structures of rat anionic trypsin complexed with the protein inhibitors APPI and BPTI" see the whole document --- -/-- | 1-133 |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex. | | |
| * Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family | | |
| Date of the actual completion of the international search 28 October 1996 | | Date of mailing of the international search report 04. 11. 96 |
| Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016 | | Authorized officer Van der Schaal, C |

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/06384

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|--|---|-----------------------|
| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| X | JOURNAL OF BIOLOGICAL CHEMISTRY 269 (35). 1994. 22137-22144. ISSN: 0021-9258, XP002015892 DENNIS M S ET AL: "Kunitz domain inhibitors of tissue factor-factor VIIa: II. Potent and specific inhibitors by competitive phage selection." cited in the application | 1,2,68 |
| Y | see table II especially page 22137, right column, fourth paragraph | 1-133 |
| X | --- JOURNAL OF BIOLOGICAL CHEMISTRY 269 (35). 1994. 22129-22136. ISSN: 0021-9258, XP002015893 DENNIS M S ET AL: "Kunitz domain inhibitors of tissue factor-factor VIIa: I. Potent inhibitors selected from libraries by phage display." cited in the application | 1,2,68 |
| Y | see table I | 1-133 |
| Y | --- JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 266, no. 31, 5 November 1991, MD US, pages 21011-21013, XP002015894 S. SINHA ET AL: "Conversion of the Alzheimer's beta-Amyloid precursor protein (APP) Kunitz domain into a potent human neutrophil elastase inhibitor" see the whole document | 1-133 |
| X | --- EP,A,0 393 431 (BAYER AG) 24 October 1990 | 1-3,68, 80 |
| Y | see the whole document | 1-133 |
| Y | --- WO,A,93 14119 (NOVONORDISK AS) 22 July 1993 see the whole document | 1-133 |
| Y | --- WO,A,93 14120 (NOVONORDISK AS) 22 July 1993 see the whole document | 1-133 |
| A | --- BIOCHEM BIOPHYS RES COMMUN 167 (2). 1990. 716-721. CODEN: BBRCA9 ISSN: 0006-291X, XP002015895 KIDO H ET AL: "PROTEASE-SPECIFICITY OF KUNITZ INHIBITOR DOMAIN OF ALZHEIMER 'S DISEASE AMYLOID PROTEIN PRECURSOR." see abstract | |
| | --- -/-- | |

3

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 96/06384

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|--|--|-----------------------|
| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| P,X | JOURNAL OF BIOLOGICAL CHEMISTRY 270 (43). 1995. 25411-25417. ISSN: 0021-9258, XP002015896 | 1 |
| Y | DENNIS M S ET AL: "Potent and Selective Kunitz Domain Inhibitors of Plasma Kallikrein Designed by Phage Display." see table I | 1-133 |
| | --- | |
| P,X | WO,A,95 23860 (GENENTECH INC) 8 September 1995 | 1-3,68, 80 |
| Y | see claims | 1-133 |
| | ----- | |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/ 06384

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 64-67, 122-125
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 64-67 and 122-125 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/06384

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|----------------------------|---------------------|
| EP-A-0393431 | 24-10-90 | DE-A- 3912638 | 31-10-90 |
| | | JP-A- 3041095 | 21-02-91 |
| WO-A-9314119 | 22-07-93 | AU-B- 671611 | 05-09-96 |
| | | AU-A- 3345793 | 03-08-93 |
| | | CA-A- 2127248 | 22-07-93 |
| | | CZ-A- 9401646 | 15-12-94 |
| | | EP-A- 0621869 | 02-11-94 |
| | | FI-A- 943231 | 06-07-94 |
| | | NO-A- 942552 | 07-09-94 |
| | | NZ-A- 246567 | 28-05-96 |
| | | ZA-A- 9300098 | 10-08-93 |
| WO-A-9314120 | 22-07-93 | AU-A- 3345893 | 03-08-93 |
| | | CA-A- 2127250 | 22-07-93 |
| | | CZ-A- 9401645 | 15-12-94 |
| | | EP-A- 0621870 | 02-11-94 |
| | | FI-A- 943232 | 06-07-94 |
| | | NO-A- 942551 | 07-09-94 |
| | | NZ-A- 246568 | 27-02-96 |
| | | ZA-A- 9300097 | 10-08-93 |
| WO-A-9523860 | 08-09-95 | NONE | |

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

CORRECTED
VERSION*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



B2

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | | |
|--|--|---|--|
| (51) International Patent Classification ⁶ : C12N 15/15, C07K 14/81, A61K 38/57 | | A3 | (11) International Publication Number: WO 97/33996 (43) International Publication Date: 18 September 1997 (18.09.97) |
| (21) International Application Number: PCT/US97/03894 (22) International Filing Date: 10 March 1997 (10.03.97) (30) Priority Data: 60/013,106 11 March 1996 (11.03.96) US 60/019,793 14 June 1996 (14.06.96) US 08/725,251 4 October 1996 (04.10.96) US (60) Parent Applications or Grants (63) Related by Continuation US 08/725,251 (CIP) Filed on 4 October 1996 (04.10.96) US 60/019,793 (CIP) Filed on 14 June 1996 (14.06.96) US 60/013,106 (CIP) Filed on 11 March 1996 (11.03.96) (71) Applicant (for all designated States except US): BAYER CORPORATION [US/US]; One Mellon Center, 500 Grant Street, Pittsburgh, PA 15219-2507 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): TAMBURINI, Paul, P. [GB/US]; 36 Misty Mountain Road, Kensington, CT 06460 | | (US). DAVIS, Gary [US/US]; 137 Tanglewood Circle, Milford, CT 06460 (US). DELARIA, Katherine, A. [US/US]; 180 West Walk Street, West Haven, CT 06516 (US). MARLOR, Christopher, W. [US/US]; 11 Robertson Drive, Bethany, CT 06524 (US). MULLER, Daniel, K. [US/US]; 253 Hemlock Hill Road, Orange, CT 06477 (US). (74) Agents: CHAO, Mark et al.; McDonnell Boehnen Hulbert & Berghoff, 300 South Wacker Drive, Chicago, IL 60606 (US). (81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 13 November 1997 (13.11.97) | |
| (54) Title: HUMAN BIKUNIN (57) Abstract <p>The instant invention provides for proteins, polypeptides, nucleic acid sequences, constructs, expression vectors, host cells, pharmaceutical compositions of, and methods for using human placental bikunin, serine protease inhibitor domains, and fragments thereof.</p> | | | |

BEST AVAILABLE COPY

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|----|--------------------------|----|--|----|--------------------------|
| AM | Armenia | GB | United Kingdom | MW | Malawi |
| AT | Austria | GE | Georgia | MX | Mexico |
| AU | Australia | GN | Guinea | NE | Niger |
| BB | Barbados | GR | Greece | NL | Netherlands |
| BE | Belgium | HU | Hungary | NO | Norway |
| BF | Burkina Faso | IE | Ireland | NZ | New Zealand |
| BG | Bulgaria | IT | Italy | PL | Poland |
| BJ | Benin | JP | Japan | PT | Portugal |
| BR | Brazil | KE | Kenya | RO | Romania |
| BY | Belarus | KG | Kyrgyzstan | RU | Russian Federation |
| CA | Canada | KP | Democratic People's Republic of Korea | SD | Sudan |
| CF | Central African Republic | KR | Republic of Korea | SE | Sweden |
| CG | Congo | KZ | Kazakhstan | SG | Singapore |
| CH | Switzerland | LI | Liechtenstein | SI | Slovenia |
| CI | Côte d'Ivoire | LK | Sri Lanka | SK | Slovakia |
| CM | Cameroon | LR | Liberia | SN | Senegal |
| CN | China | LT | Lithuania | SZ | Swaziland |
| CS | Czechoslovakia | LU | Luxembourg | TD | Chad |
| CZ | Czech Republic | LV | Latvia | TG | Togo |
| DE | Germany | MC | Monaco | TJ | Tajikistan |
| DK | Denmark | MD | Republic of Moldova | TT | Trinidad and Tobago |
| EE | Estonia | MG | Madagascar | UA | Ukraine |
| ES | Spain | ML | Mali | UG | Uganda |
| FI | Finland | MN | Mongolia | US | United States of America |
| FR | France | MR | Mauritania | UZ | Uzbekistan |
| GA | Gabon | | | VN | Viet Nam |

Human Bikunin

Field of the Invention

The compositions of the invention relate to the field of proteins which
5 inhibit serine protease activity. The invention also relates to the field of nucleic acid constructs, vectors and host cells for producing serine protease inhibiting proteins, pharmaceutical compositions containing the protein, and methods for their use.

10 Background of the Invention

Problem Addressed

Blood loss is a serious complication of major surgeries such as open heart surgery and other complicated procedures. Cardiac surgery patients account for a significant proportion of transfused donor blood. Blood transfusion
15 carries risks of disease transmission and adverse reactions. In addition, donor blood is expensive and demands often exceed supply. Pharmacological methods for reducing blood loss and the resultant need for transfusion have been described (reviewed by Scott et al., Ann. Thorac. Surg. 50: 843-851, 1990).

20 *Protein Serine Protease Inhibitor*

Aprotinin, a bovine serine protease inhibitor of the Kunitz family is the active substance in the medicament Trasylol®. Aprotinin (Trasylol®) has been reported as being effective in reducing perioperative blood loss (Royston et al., Lancet ii: 1289-1291, 1987; Dietrich et al., Thorac. Cardiovasc. Surg. 37: 92-98,
25 1989; Fraedrich et al., Thorac. Cardiovasc. Surg. 37: 89-91, 1989); W. van Oeveren et al. (1987), Ann Thorac. Surg. 44, pp 640-645; Bistrup et al., (1988) Lancet I, 366-367), but adverse effects, including hypotension and flushing (Bohrer et al., Anesthesia 45: 853-854, 1990) and allergic reactions (Dietrich et al., Supra) have been reported. Use of aprotinin in patients previously exposed
30 to it is not recommended (Dietrich et al., Supra). Trasylol® has also been used for the treatment of hyperfibrinolytic hemorrhages and traumatic hemorrhagic shock.

Aprotinin is known to inhibit several serine proteases including trypsin, chymotrypsin, plasmin and kallikrein, and is used therapeutically in the
35 treatment of acute pancreatitis, various states of shock syndrome, hyperfibrinolytic hemorrhage and myocardial infarction (Trapnell et al., (1974) Brit J. Surg. 61: 177; J. McMichan et al., (1982) Circulatory Shock 9: 107; Auer et

al., (1979)Acta Neurochir. 49: 207; Sher (1977) Am J. Obstet. Gynecol. 129: 164; Schneider (1976) , Arzneim.-Firsch. 26: 1606). It is generally thought that Trasylol® reduces blood loss *in vivo* through inhibition of kallikrein and plasmin. It has been found that aprotinin (3-58, Arg15, Ala17, Ser42) exhibits
5 improved plasma kallikrein inhibitory potency as compared to native aprotinin itself (WO 89/10374).

Problems With Aprotinin

Because aprotinin is of bovine origin, there is a finite risk of inducing
10 anaphylaxis in human patients upon re-exposure to the drug. Thus, a human functional equivalent to aprotinin, by virtue of a lower risk of anaphylaxis, would be most useful and desirable to have.

Aprotinin is also nephrotoxic in rodents and dogs when administered repeatedly at high dose (Bayer, Trasylol®, Inhibitor of proteinase; Glasser et al.,
15 in "Verhandlungen der Deutschen Gesellschaft fur Innere Medizin, 78. Kongress", Bergmann, Munchen, 1972 pp. 1612-1614). One hypothesis ascribes this effect to the accumulation of aprotinin in the negatively charged proximal tubules of the kidney, due to its high net positive charge (WO 93/14120).

Accordingly, an object of the present invention is to identify human
20 proteins with functional activity similar to aprotinin. It was also an object of the instant invention to identify human proteins, that would be less charged, yet exhibit the same, highly similar, or improved protease specificities as found for aprotinin, especially with respect to the potency of plasmin and kallikrein inhibition. Such inhibitors could then be used repeatedly as medicaments in
25 human patients with reduced risk of adverse immune response and reduced nephrotoxicity.

Brief Summary of the Invention

The instant invention provides for a purified human serine protease
30 inhibitor which can specifically inhibit kallikrein, that has been isolated from human placental tissue via affinity chromatography.

The instant invention provides a newly identified human protein herein called human placental bikunin that contains two serine protease inhibitor domains of the Kunitz class. In one particular embodiment, the instant
35 invention embodies a protein having the amino acid sequence:

ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
 YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
 NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
 ACMLRCFRQQ ENPPLPLGSK VVVLGAVS 179

5 (SEQ ID NO: 1)

In a preferred embodiment the instant invention provides for native human placental bikunin protein having the amino acid sequence:

10 ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
 YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
 NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
 ACMLRCFRQQ ENPPLPLGSK 170

(SEQ ID NO: 52)

15

In one aspect, the biological activity of the protein of the instant invention is that it can bind to and substantially inhibit the biological activity of trypsin, human plasma and tissue kallikreins, human plasmin and Factor XIIa. In a preferred embodiment, the present invention provides for a native human placental bikunin protein, in glycosylated form. In a further embodiment the instant invention encompasses native human bikunin protein which has been formed such that it contains at least one cysteine-cysteine disulfide bond. In a preferred embodiment, the protein contains at least one intra-chain cysteine-cysteine disulfide bond formed between a pair of cysteines selected from the group consisting of CYS11-CYS61, CYS20-CYS44, CYS36-CYS57, CYS106-CYS156, CYS115-CYS139, and CYS131-CYS152, wherein the cysteines are numbered according to the amino acid sequence of native human placental bikunin. One of ordinary skill will recognize that the protein of the instant invention may fold into the proper three-dimensional conformation, such that the biological activity of native human bikunin is maintained, where none, one or more, or all of the native intra-chain cysteine-cysteine disulfide bonds are present. In a most preferred embodiment, the protein of the instant invention is properly folded and is formed with all of the proper native cysteine-cysteine disulfide bonds.

35 Active protein of the instant invention can be obtained by purification from human tissue, such as placenta, or via synthetic protein chemistry techniques, as illustrated by the Examples below. It is also understood that the

protein of the instant invention may be obtained using molecular biology techniques, where self-replicating vectors are capable of expressing the protein of the instant invention from transformed cells. Such protein can be made as non-secreted, or secreted forms from transformed cells. In order to facilitate secretion from transformed cells, to enhance the functional stability of the translated protein, or to aid folding of the bikunin protein, certain signal peptide sequences may be added to the NH₂-terminal portion of the native human bikunin protein.

In one embodiment, the instant invention thus provides for the native human bikunin protein with at least a portion of the native signal peptide sequence intact. Thus one embodiment of the invention provides for native human bikunin with at least part of the signal peptide, having the amino acid sequence:

15 AGSFLAWLGSLLLSGVLA -1
 ADRERSIHDFCLVSKVVGRCRASMPRWYNVTDGSCQLFVYGGCDGNSNN 50
 YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDSEDHSSDMF 100
 NYEEYCTANAVTGPCRASFPWYFDVERNSCNNFIYGGCRGNKNSYRSEE 150
 ACMLRCFRQQENPPLPLGSKVVVLAVS 179
 20 (SEQ ID NO: 2)

In a preferred embodiment the instant invention provides for a native human placental bikunin protein with part of the leader sequence intact, having the amino acid sequence of SEQ ID NO: 52 with an intact leader segment having the amino acid sequence:

25 MAQLCGL RRSRAFLALL GSLLLSGVLA -1
 (SEQ ID NO: 53)

In another embodiment, the instant invention provides for bikunin protein with part of the leader sequence intact, having the amino acid sequence of SEQ ID NO: 52 with the intact leader segment having the amino acid sequence:

30 MLR AEADGVSRLL GSLLLSGVLA -1
 (SEQ ID NO: 54)

35 In a preferred numbering system used herein the amino acid numbered +1 is assigned to the NH₂-terminus of the amino acid sequence for native

human placental bikunin. One will readily recognize that functional protein fragments can be derived from native human placental bikunin, which will maintain at least part of the biological activity of native human placental bikunin, and act as serine protease inhibitors.

5 In one embodiment, the protein of the instant invention comprises a fragment of native human placental bikunin, which contains at least one functional Kunitz-like domain, having the amino acid sequence of native human placental bikunin amino acids 7-159, hereinafter called "bikunin (7-159)". Thus the instant invention embodies a protein having the amino acid
10 sequence:

```

IHDFCLVSKVVGRCRASMPRWYNVTDGSCQLFVYGGCDGNSNN      50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDSEDHSSDMF 100
NYEEYCTANAVTGPCRASFPWYFDVERNSCNNFIYGGCRGNKNSYRSEE 150
15 ACMLRCFRQ                                           159
(SEQ ID NO: 3)

```

where the amino acid numbering corresponds to that of the amino acid sequence of native human placental bikunin. Another functional variant of this
20 embodiment can be the fragment of native human placental bikunin, which contains at least one functional Kunitz-like domain, having the amino acid sequence of native human placental bikunin amino acids 11-156, bikunin (11-156)

```

25 CLVSKVVGRCRASMPRWYNVTDGSCQLFVYGGCDGNSNN      50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDSEDHSSDMF 100
NYEEYCTANAVTGPCRASFPWYFDVERNSCNNFIYGGCRGNKNSYRSEE 150
ACMLRC                                           156
30 (SEQ ID NO: 50).

```

One can recognize that the individual Kunitz-like domains are also fragments of the native placental bikunin. In particular, the instant invention provides for a protein having the amino acid sequence of a first Kunitz-like domain consisting of the amino acid sequence of native human placental
35 bikunin amino acids 7-64, hereinafter called "bikunin (7-64)". Thus in one embodiment the instant invention encompasses a protein which contains at least one Kunitz-like domain having the amino acid sequence:

IHDFCLVSKVVGRCRASMPRWYNVTDGSCQLFVYGGCDGNSNN 50
 YLTKEECLKKCATV 64
 (SEQ ID NO: 4)

5

where the amino acid numbering corresponds to that of the amino acid sequence of native human placental bikunin. Another form of the protein of the instant invention can be a first Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 11-61, "bikunin (11-10 61)" having the amino acid sequence:

CLVSKVVGRCRASMPRWYNVTDGSCQLFVYGGCDGNSNN 50
 YLTKEECLKKC 61
 (SEQ ID NO: 5)

15

The instant invention also provides for a protein having the amino acid sequence of a Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 102-159, hereinafter called "bikunin (102-159)". Thus one embodiment the instant invention encompasses a 20 protein which contains at least one Kunitz-like domain having the amino acid sequence:

YEEYCTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE 150
 ACMLRCFRQ 159
 25 (SEQ ID NO: 6)

where the amino acid numbering corresponds to that of the amino acid sequence of native human placental bikunin. Another form of this domain can be a Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 106-156, "bikunin (106-156)" having the amino acid sequence:

CTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE 150
 ACMLRC 156
 35 (SEQ ID NO: 7)

Thus one of ordinary skill will recognize that fragments of the native

human bikunin protein can be made which will retain at least some of the native protein biological activity. Such fragments can also be combined in different orientations or multiple combinations to provide for alternative proteins which retain some of, the same, or more biological activity of the native human bikunin protein.

One will readily recognize that biologically active protein of the instant invention may comprise one or more of the instant Kunitz-like domains in combination with additional Kunitz-like domains from other sources. Biologically active protein of the instant invention may comprise one or more of the instant Kunitz-like domains in combination with additional protein domains from other sources with a variety of biological activities. The biological activity of the protein of the instant invention can be combined with that of other known protein or proteins to provide for multifunctional fusion proteins having predictable biological activity. Thus, in one embodiment, the instant invention encompasses a protein which contains at least one amino acid sequence segment the same as, or functionally equivalent to the amino acid sequence of either SEQ ID NO: 5 or SEQ ID NO: 7.

An open reading frame which terminates at an early stop codon can still code for a functional protein. The instant invention encompasses such alternative termination, and in one embodiment provides for a protein of the amino acid sequence:

ADRERSIHDFCLVSKVVGRCPASMPRWVYNVTDGSCQLFVYGGCDGNSNN 50

YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDS 92

(SEQIDNO:8)

In one embodiment, the instant invention provides for substantially purified, or recombinantly produced native human bikunin protein with an intact segment of the leader sequence, and at least a portion of the native transmembrane region intact. Thus one embodiment of the invention provides for native human bikunin, with an intact leader sequence, and with at least part of the transmembrane domain (underlined), having an amino acid sequence selected from:

| | | | | |
|----|-------------------|-----------------------|-----------------------|-----|
| | 1) EST | MLR AEADGVSRL | GSLLLSGVLA | -1 |
| | 2) PCR | MAQLCGL RRSRAFLALL | GSLLLSGVLA | -1 |
| | 3) λ cDNA | MAQLCGL RRSRAFLALL | GSLLLSGVLA | -1 |
| 5 | 1) ADRERSIHDF | CLVSKVVGRC RASMPRWYN | VTDGSCQLFV YGGCDGNSNN | 50 |
| | 2) ADRERSIHDF | CLVSKVVGRC RASMPRWYN | VTDGSCQLFV YGGCDGNSNN | 50 |
| | 3) ADRERSIHDF | CLVSKVVGRC RASMPRWYN | VTDGSCQLFV YGGCDGNSNN | 50 |
| 10 | 1) YLTKEECLKK | CATVTENATG DLATSRNAAD | SSVPSAPRRQ DSEDHSSDMF | 100 |
| | 2) YLTKEECLKK | CATVTENATG DLATSRNAAD | SSVPSAPRRQ DSEDHSSDMF | 100 |
| | 3) YLTKEECLKK | CATVTENATG DLATSRNAAD | SSVPSAPRRQ DSEDHSSDMF | 100 |
| | 1) NYEEYCTANA | VTGPCRASFP RWYFDVERNS | CNNFIYGGCR GNKNSYRSEE | 150 |
| | 2) NYEEYCTANA | VTGPCRASFP RWYFDVERNS | CNNFIYGGCR GNKNSYRSEE | 150 |
| 15 | 3) NYEEYCTANA | VTGPCRASFP RWYFDVERNS | CNNFIYGGCR GNKNSYRSEE | 150 |
| | 1) ACMLRCFRQQ | ENPPLPLGSK VVVLAGLFVM | VLILFLGASM VYLIRVARRN | 200 |
| | 2) ACMLRCFRQQ | ENPPLPLGSK VVVLAGLFVM | VLILFLGASM VYLIRVARRN | 200 |
| | 3) ACMLRCFRQQ | ENPPLPLGSK VVVLAGLFVM | VLILFLGASM VYLIRVARRN | 200 |
| 20 | 1) QERALRTVWS | SGDDKEQLVK NTYVL | | 225 |
| | 2) QERALRTVWS | FGD | | 213 |
| | 3) QERALRTVWS | SGDDKEQLVK NTYVL | | 225 |

- 25 where sequence 1) is EST derived consensus SEQ ID NO: 45, 2) is PCR clone SEQ ID NO: 47, and 3) is lambda cDNA clone SEQ ID NO: 49. In a preferred embodiment a protein of the instant invention comprises one of the amino acid sequence of SEQ ID NO: 45, 47 or 49 wherein the protein has been cleaved in the region between the end of the last Kunitz domain and the transmembrane region.
- 30

The instant invention also embodies the protein wherein the signal peptide is deleted. Thus the instant invention provides for a protein having the amino acid sequence of SEQ ID NO: 52 continuous with a transmembrane amino acid sequence:

| | | | |
|----|-----|----------------------------------|-----|
| 35 | EST | VVVLAGLFVM VLILFLGASM VYLIRVARRN | 200 |
| | EST | QERALRTVWS SGDDKEQLVK NTYVL | 225 |
| | | (SEQ ID NO: 69) | |

a transmembrane amino acid sequence:

| | | | |
|----|-----|----------------------------------|-----|
| | PCR | VVVLAGLFVM VLILFLGASM VYLIRVARRN | 200 |
| 40 | PCR | QERALRTVWS FGD | 213 |
| | | (SEQ ID NO: 68) | |

or a transmembrane amino acid sequence:

| | | | |
|----|----------------|----------------------------------|-----|
| | λ cDNA | VVVLAGLFVM VLILFLGASM VYLIRVARRN | 200 |
| | λ cDNA | QERALRTVWS SGDDKEQLVK NTYVL | 225 |
| 45 | | (SEQ ID NO: 67) | |

The protein amino acid sequences of the instant invention clearly teach one of the art the appropriate nucleic acid sequences which can be used in molecular biology techniques to produce the proteins of the instant invention. Thus, one embodiment of the instant invention provides for a nucleic acid sequence which encodes for a human bikunin having the consensus DNA sequence of Figure 3 (SEQ ID NO: 9), which translates into the amino acid sequence for native human placental bikunin sequence of Figure 3 (SEQ ID NO: 10). In another embodiment, the instant invention provides for a consensus nucleic acid sequence of Figure 4C (SEQ ID NO: 51) which encodes for an amino acid sequence of Figure 4D (SEQ ID NO: 45).

In a preferred embodiment, the instant invention provides for a nucleic acid sequence which encodes for native human placental bikunin having the DNA sequence of Figure 4F (SEQ ID NO: 48) which encodes for the protein sequence of SEQ ID NO: 49. In another embodiment, the instant invention provides for a nucleic acid sequence of Figure 4E (SEQ ID NO: 46) which encodes for a protein sequence of SEQ ID NO: 47.

One can easily recognize that certain allelic mutations, and conservative substitutions made in the nucleic acid sequence can be made which will still result in a protein amino acid sequence encompassed by the instant invention. One of skill in the art can recognize that certain natural allelic mutations of the protein of the instant invention, and conservative substitutions of amino acids in the protein of the instant invention will not significantly alter the biological activity of the protein, and are encompassed by the instant invention.

The instant invention also provides for pharmaceutical compositions containing human placental bikunin and fragments thereof that are useful for the reduction of perioperative blood loss in a patient undergoing surgery.

The present invention also provides methods for reducing perioperative blood loss in a patient undergoing surgery, wherein an effective amount of the disclosed human serine protease inhibitors of the present invention in a biologically compatible vehicle is administered to the patient.

The present invention also provides for variants of placental bikunin, and the specific Kunitz domains described above, that contain amino acid substitutions that alter the protease specificity. Preferred sites of substitution are indicated below as positions Xaa¹ through Xaa³² in the amino acid sequence for native placental bikunin. Substitutions at Xaa¹ through Xaa¹⁶ are also preferred for variants of bikunin (7-64), while substitutions at Xaa¹⁷ through Xaa³² are preferred for variants of bikunin (102-159).

Thus the present invention embodies protein having an amino acid sequence:

| | | |
|----|---|-----|
| | Ala Asp Arg Glu Arg Ser Ile Xaa ¹ Asp Phe | 10 |
| | Cys Leu Val Ser Lys Val Xaa ² Gly Xaa ³ Cys | 20 |
| 5 | Xaa ⁴ Xaa ⁵ Xaa ⁶ Xaa ⁷ Xaa ⁸ Xaa ⁹ Trp Trp Tyr Asn | 30 |
| | Val Thr Asp Gly Ser Cys Gln Leu Phe Xaa ¹⁰ | 40 |
| | Tyr Xaa ¹¹ Gly Cys Xaa ¹² Xaa ¹³ Xaa ¹⁴ Ser Asn Asn | 50 |
| | Tyr Xaa ¹⁵ Thr Lys Glu Glu Cys Leu Lys Lys | 60 |
| | Cys Ala Thr Xaa ¹⁶ Thr Glu Asn Ala Thr Gly | 70 |
| 10 | Asp Leu Ser Thr Ser Arg Asn Ala Ala Asp | 80 |
| | Ser Ser Val Pro Ser Ala Pro Arg Arg Gln | 90 |
| | Asp Ser Glu His Asp Ser Ser Asp Met Phe | 100 |
| | Asn Tyr Xaa ¹⁷ Glu Tyr Cys Thr Ala Asn Ala | 110 |
| | Val Xaa ¹⁸ Gly Xaa ¹⁹ Cys Xaa ²⁰ Xaa ²¹ Xaa ²² Xaa ²³ Xaa ²⁴ | 120 |
| 15 | Xaa ²⁵ Trp Tyr Phe Asp Val Glu Arg Asn Ser | 130 |
| | Cys Asn Asn Phe Xaa ²⁶ Tyr Xaa ²⁷ Gly Cys Xaa ²⁸ | 140 |
| | Xaa ²⁹ Xaa ³⁰ Lys Asn Ser Tyr Xaa ³¹ Ser Glu Glu | 150 |
| | Ala Cys Met Leu Arg Cys Phe Arg Xaa ³² Gln | 160 |
| | Glu Asn Pro Pro Leu Pro Leu Gly Ser Lys | 170 |
| 20 | Val Val Val Leu Ala Gly Ala Val Ser | 179 |
| | (SEQ ID NO: 11). | |

where Xaa¹ - Xaa³² each independently represents a naturally occurring amino acid residue except Cys, with the proviso that at least one of the amino acid residues Xaa¹-Xaa³² is different from the corresponding amino acid residue of the native sequence.

In the present context, the term "naturally occurring amino acid residue" is intended to indicate any one of the 20 commonly occurring amino acids, i.e., Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.

By substituting one or more amino acids in one or more of the positions indicated above, it may be possible to change the inhibitor specificity profile of native placental bikunin or that of the individual Kunitz-like domains, bikunin(7-64) or bikunin (102-159) so that it preferentially inhibits other serine proteases such as, but not limited to, the enzymes of the complement cascade, TF/FVIIa, FXa, thrombin, neutrophil elastase, cathepsin G or proteinase-3.

Examples of preferred variants of placental bikunin include those

wherein Xaa¹ is an amino acid residue selected from the group consisting of His, Glu, Pro, Ala, Val or Lys, in particular wherein Xaa¹ is His or Pro; or wherein Xaa² is an amino acid residue selected from the group consisting of Val, Thr, Asp, Pro, Arg, Tyr, Glu, Ala, Lys, in particular wherein Xaa² is Val or Thr; or wherein Xaa³ is an amino acid residue selected from the group consisting of Arg, Pro, Ile, Leu, Thr, in particular wherein Xaa³ is Arg or Pro; or wherein Xaa⁴ is an amino acid residue selected from the group consisting of Arg, Lys and Ser, Gln, in particular wherein Xaa⁴ is Arg or Lys; or wherein Xaa⁵ is an amino acid residue selected from the group consisting of Ala, Gly, Asp, Thr, in particular wherein Xaa⁵ is Ala; or wherein Xaa⁶ is an amino acid residue selected from the group consisting of Ser, Ile, Tyr, Asn, Leu, Val, Arg, Phe, in particular wherein Xaa⁶ is Ser or Arg; or wherein Xaa⁷ is an amino acid residue selected from the group consisting of Met, Phe, Ile, Glu, Leu, Thr and Val, in particular wherein Xaa⁷ is Met or Ile; or wherein Xaa⁸ is an amino acid residue selected from the group consisting of Pro, Lys, Thr, Gln, Asn, Leu, Ser or Ile, in particular wherein Xaa⁸ is Pro or Ile; or wherein Xaa⁹ is an amino acid residue selected from the group consisting of Arg, Lys or Leu, in particular wherein Xaa⁹ is Arg; or wherein Xaa¹⁰ is an amino acid residue selected from the group consisting of Val, Ile, Lys, Ala, Pro, Phe, Trp, Gln, Leu and Thr, in particular wherein Xaa¹⁰ is Val; or wherein Xaa¹¹ is an amino acid residue selected from the group consisting of Gly, Ser and Thr, in particular wherein Xaa¹¹ is Gly; or wherein Xaa¹² is an amino acid residue selected from the group consisting of Asp, Arg, Glu, Leu, Gln, Gly, in particular wherein Xaa¹² is Asp or Arg; or wherein Xaa¹³ is an amino acid residue selected from the group consisting of Gly and Ala; or wherein Xaa¹⁴ is an amino acid residue selected from the group consisting of Asn or Lys; or wherein Xaa¹⁵ is an amino acid residue selected from the group consisting of Gly, Asp, Leu, Arg, Glu, Thr, Tyr, Val, and Lys, in particular wherein Xaa¹⁵ is Leu or Lys; or wherein Xaa¹⁶ is an amino acid residue selected from the group consisting of Val, Gln, Asp, Gly, Ile, Ala, Met, and Val, in particular wherein Xaa¹⁶ is Val or Ala; or wherein Xaa¹⁷ is an amino acid residue selected from the group consisting of His, Glu, Pro, Ala, Lys and Val, in particular wherein Xaa¹⁷ is Glu or Pro; or wherein Xaa¹⁸ is an amino acid residue selected from the group consisting of Val, Thr, Asp, Pro, Arg, Tyr, Glu, Ala or Lys, in particular wherein Xaa¹⁸ is Thr; or wherein Xaa¹⁹ is an amino acid residue selected from the group consisting of Arg, Pro, Ile, Leu or Thr, in particular wherein Xaa¹⁹ is Pro; or wherein Xaa²⁰ is an amino acid residue selected from the group consisting of Arg, Lys, Gln and

Ser, in particular wherein Xaa²⁰ is Arg or Lys; or wherein Xaa²¹ is an amino acid residue selected from the group consisting of Ala, Asp, Thr or Gly ; in particular wherein Xaa²¹ is Ala; or wherein Xaa²² is an amino acid residue selected from the group consisting of Ser, Ile, Tyr, Asn, Leu, Val, Arg or Phe, in particular wherein Xaa²² is Ser or Arg ; or wherein Xaa²³ is an amino acid residue selected from the group consisting of Met, Phe, Ile, Glu, Leu, Thr and Val, in particular wherein Xaa²³ is Phe or Ile; or wherein Xaa²⁴ is an amino acid residue selected from the group consisting of Pro, Lys, Thr, Asn, Leu, Gln, Ser or Ile, in particular wherein Xaa²⁴ is Pro or Ile; or wherein Xaa²⁵ is an amino acid residue selected from the group consisting of Arg, Lys or Leu, in particular wherein Xaa²⁵ is Arg; or wherein Xaa²⁶ is an amino acid residue selected from the group consisting of Val, Ile, Lys, Leu, Ala, Pro, Phe, Gln, Trp and Thr, in particular wherein Xaa²⁶ is Val or Ile; or wherein Xaa²⁷ is an amino acid residue selected from the group consisting of Gly, Ser and Thr, in particular wherein Xaa²⁷ is Gly; or wherein Xaa²⁸ is an amino acid residue selected from the group consisting of Asp, Arg, Glu, Leu, Gly or Gln, in particular wherein Xaa²⁸ is Arg; or wherein Xaa²⁹ is an amino acid residue selected from the group consisting of Gly and Ala; or wherein Xaa³⁰ is an amino acid residue selected from the group consisting of Asn or Lys; or wherein Xaa³¹ is an amino acid residue selected from the group consisting of Gly, Asp, Leu, Arg, Glu, Thr, Tyr, Val, and Lys, in particular wherein Xaa³¹ is Arg or Lys; or wherein Xaa³² is an amino acid residue selected from the group consisting of Val, Gln, Asp, Gly, Ile, Ala, Met, and Thr, in particular wherein Xaa³² is Gln or Ala.

Description of the Drawings

The invention will be better understood from a consideration of the following detailed description and claims, taken in conjunction with the drawings, in which:

Figure 1 depicts the nucleotide sequence of EST R35464 (SEQ ID NO: 12) and the translation of this DNA sequence (SEQ ID NO: 13) which yielded an open reading frame with some sequence similarity to aprotinin. The translation product contains 5 of the 6 cysteines in the correct spacing that is characteristic for Kunitz-like inhibitor domains (indicated in bold). The position normally occupied by the remaining cysteine (at codon 38) contained instead a phenylalanine (indicated by an asterisk).

Figure 2 depicts the nucleotide sequence of EST R74593 (SEQ ID NO: 14),

and the translation of this DNA sequence (SEQ ID NO: 15) which yielded an open reading frame with homology to the Kunitz class of serine protease inhibitor domains. The translation product contained 6 cysteines in the correct spacing that is characteristic for Kunitz-like inhibitor domains (indicated in bold). However, this reading frame sequence includes stop codons at codon 3 and 23.

Figure 3 depicts a deduced nucleic acid sequence of human placental bikunin (SEQ ID NO: 9) labeled "consensus" and matched with the translated protein amino acid sequence labeled "translated" (SEQ ID NO: 10). Also as comparison are shown the nucleic acid sequence for ESTs H94519 (SEQ ID NO: 16), N39798 (SEQ ID NO: 17), R74593 (SEQ ID NO: 14) and R35464 (SEQ ID NO: 12). The underlined nucleotides in the consensus sequence correspond to the site of PCR primers described in the Examples. Underlined amino acids in the translated consensus sequence are residues whose identity have been confirmed by amino acid sequencing of purified native human placental bikunin. Nucleotide and amino acid code are standard single letter code, "N" in the nucleic acid code indicates an unassigned nucleic acid, and "*" indicates a stop codon in the amino acid sequence.

Figure 4A depicts the original overlay of a series of ESTs with some nucleic acid sequence homology to ESTs encoding human placental bikunin, or portions thereof. Shown for reference are the relative positions of bikunin (7-64) and bikunin (102-159), labeled KID1 and KID2 respectively.

Figure 4B depicts a subsequent more comprehensive EST overlay incorporating additional ESTs. Numbers on the upper X-axis refer to length in base pairs, starting at the first base from the most 5' EST sequence. The length of each bar is in proportion to the length in base pairs of the individual ESTs including gaps. The EST accession numbers are indicated to the right of their respective EST bars.

Figure 4C depicts the corresponding alignment of the oligonucleotide sequences of each of the overlapping ESTs shown schematically in Figure 4B. The upper sequence (SEQ ID NO: 51) labeled bikunin represents the consensus oligonucleotide sequence derived from the overlapping nucleotides at each position. The numbers refer to base-pair position within the EST map. The oligonucleotides in EST R74593 that are bold underlined (at map positions 994 and 1005) are base insertions observed in R74593 that were consistently absent in each of the other overlapping ESTs.

Figure 4D depicts the amino acid translation of the consensus

oligonucleotide sequence for bikunin depicted in Figure 4C (SEQ ID NO: 45).

Figure 4E depicts the nucleotide sequence (SEQ ID NO: 46) and corresponding amino acid translation (SEQ ID NO: 47) of a placental bikunin encoding sequence that was derived from a human placental cDNA library by PCR-based amplification.

Figure 4F depicts the nucleotide sequence (SEQ ID NO: 48) and corresponding amino acid translation (SEQ ID NO: 49) of a native human placental bikunin encoding clone that was isolated from a human placental lambda cDNA library by colony hybridization.

Figure 4G compares the alignment of the amino acid translated oligonucleotide sequences for placental bikunin obtained by EST overlay (SEQ ID NO: 45), PCR based cloning (SEQ ID NO: 47), and conventional lambda colony hybridization (SEQ ID NO: 49).

Figure 5 shows a graph of purification of human placental bikunin from placental tissue after Superdex 75 Gel-Filtration. The plot is an overlay of the protein elution profile as measured by OD 280 nm (solid line), activity of eluted protein in a trypsin inhibition assay (% inhibition shown by circles), and activity of eluted protein in a kallikrein inhibition assay (% inhibition shown by squares).

Figure 6 shows a graph which plots the purification of human placental bikunin from placental tissue using C18 Reverse-Phase Chromatography. The plot is an overlay of the protein elution profile as measured by OD 215 nm (solid line), activity of eluted protein in a trypsin inhibition assay (% inhibition shown by circles), and activity of eluted protein in a kallikrein inhibition assay (% inhibition shown by squares).

Figure 7 depicts a silver stained SDS-PAGE gel of highly purified placental bikunin (lane 2), and a series of molecular size marker proteins (lane 1) of the indicated sizes in kilodaltons. Migration was from top to bottom.

Figure 8 shows the amount of trypsin inhibitory activity present in the cell-free fermentation broth from the growth of yeast strains SC101 (panel 8A) or WHL341 (panel 8B) that were stably transformed with a plasmid (pS604) that directs the expression of placental bikunin (102-159).

Figure 9 shows both a silver stained SDS-PAGE (left panel) and a Western blot with anti-placental bikunin (102-159) pAb (right panel) of cell-free fermentation broth from the growth of yeast strain SC101 (recombinants 2.4 and 2.5) that was stably transformed with a plasmid directing the expression of either bovine aprotinin, or placental bikunin (102-159). Migration was from top

to bottom.

Figure 10 is a photograph which shows a silver stained SDS-PAGE of highly purified placental bikunin (102-159) (lane 2) and a series of molecular size marker proteins (lane 1) of the indicated sizes in Kilodaltons. Migration was from top to bottom.

Figure 11 is a photograph which shows the results of Northern blots of mRNA from various human tissues that was hybridized to a ³²P labeled cDNA probe encoding either placental bikunin (102-159) (panel 11A) or encoding placental bikunin (1-213) (panel 11B). Migration was from top to bottom. The numbers to the right of each blot refer to the size in kilobases of the adjacent RNA markers. The organs from which mRNA was derived is described under each lane of the blot.

Figure 12 depicts an immunoblot of placental derived placental bikunin with rabbit antiserum raised against either synthetic reduced placental bikunin (7-64) (panel A) or 102-159 (panel B). For each panel, contents were: molecular size markers (lanes 1); native placental bikunin isolated from human placenta (lanes 2); synthetic placental bikunin (7-64) (lanes 3) and synthetic placental bikunin (102-159) (lanes 4). Tricine 10-20% SDS-PAGE gels were blotted and developed with protein A-purified primary polyclonal antibody (8 ug IgG in 20 ml 0.1% BSA/Tris-buffered saline (pH 7.5), followed by alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. Migration was from top to bottom.

Figure 13 depicts a Coomassie Blue stained 10-20% Tricine SDS-PAGE gel of 3 micrograms of highly purified placental bikunin (1-170) derived from a baculovirus / Sf9 expression system (lane 2). Lane 1 contains molecular size markers. Migration was from top to bottom.

Figure 14 depicts a comparison of the effect of increasing concentrations of either Sf9-derived human placental bikunin (1-170) (filled circles), synthetic placental bikunin (102-159) (open circles), or aprotinin (open squares) on the activated partial thromboplastin time of human plasma. Clotting was initiated with CaCl₂. The concentration of proteins are plotted versus the -fold prolongation in clotting time. The uninhibited clotting time was 30.8 seconds.

Detailed Description of the Invention

The present invention encompasses a newly identified human protein herein called human placental bikunin that contains two serine protease inhibitor domains of the Kunitz class. The instant invention also encompasses

pharmaceutical compositions containing placental bikunin and fragments thereof that are useful for the reduction of perioperative blood loss in a patient undergoing surgery, or with major trauma.

5 The present invention also provides methods for reducing perioperative blood loss in a patient undergoing surgery or due to major trauma, wherein an effective amount of the disclosed human serine protease inhibitors of the present invention, in a biologically compatible vehicle, is administered to the patient.

10 A preferred application for placental bikunin, isolated domains, and other variants is for the reduction of blood loss resulting from trauma or surgery that has the potential for loss of large volumes of blood. These methods and compositions reduce or eliminate the need for whole donor blood or blood products, thereby reducing the risk of infection and other adverse side effects, as well as the cost of surgery. The methods are thus useful in reducing
15 blood loss in normal patients, i.e., those not suffering from inborn or other pre-operative deficiencies in coagulation factors. The reduction in blood loss is seen as a reduction in blood loss during surgery, as reduced post surgical drainage or both. Preferred surgical applications include but are not limited to use in thoracic and abdominal surgery, total and partial hip replacement surgeries and surgeries to treat a patient having an epithelial lesion of the eye. Preferred
20 thoracic surgical procedures include but are not limited to aortocoronary bypass, excision of cardiac and aortic aneurysms, and surgery for esophageal varices, and coronary artery bypass surgery. Preferred abdominal surgeries include but are not limited to liver transplants, radical prostatectomy, surgery
25 for diverticulitis of colon, tumor debulking, surgery on the abdominal aorta and surgery for duodenal ulcers, and repair of liver or spleen trauma. Preferred use for the treatment of trauma include but are not limited to the use in stabilization of severely injured patients at accident sites suffering from e.g., limb loss or major thoracic /abdominal wounds. In case of use for the
30 reduction of blood loss resulting from surgery it is preferred to administer the placental bikunin, isolated domains, or other variant prior to and during surgery, whereas in case of use in trauma settings the placental bikunin variant, isolated domain or other variant is to be administered as rapidly as possible following injury, and should be contained on emergency vehicles traveling to
35 the accident sites.

Factor XII (also known as Hageman Factor) is a serine protease that is found in the circulation in a zymogen form (80 kD) at approximately 29-40

**CORRECTED
VERSION***

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | | |
|--|--|-----------|--|
| (51) International Patent Classification ⁶ : C12N 15/15, C07K 14/81, A61K 38/57 | | A3 | (11) International Publication Number: WO 97/33996 (43) International Publication Date: 18 September 1997 (18.09.97) |
| (21) International Application Number: PCT/US97/03894 (22) International Filing Date: 10 March 1997 (10.03.97) (30) Priority Data: 60/013,106 11 March 1996 (11.03.96) US 60/019,793 14 June 1996 (14.06.96) US 08/725,251 4 October 1996 (04.10.96) US (60) Parent Applications or Grants (63) Related by Continuation US 08/725,251 (CIP) Filed on 4 October 1996 (04.10.96) US 60/019,793 (CIP) Filed on 14 June 1996 (14.06.96) US 60/013,106 (CIP) Filed on 11 March 1996 (11.03.96) (71) Applicant (for all designated States except US): BAYER CORPORATION [US/US]; One Mellon Center, 500 Grant Street, Pittsburgh, PA 15219-2507 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): TAMBURINI, Paul, P. [GB/US]; 36 Misty Mountain Road, Kensington, CT 06460 | | | (US). DAVIS, Gary [US/US]; 137 Tanglewood Circle, Milford, CT 06460 (US). DELARIA, Katherine, A. [US/US]; 180 West Walk Street, West Haven, CT 06516 (US). MARLOR, Christopher, W. [US/US]; 11 Robertson Drive, Bethany, CT 06524 (US). MULLER, Daniel, K. [US/US]; 253 Hemlock Hill Road, Orange, CT 06477 (US). (74) Agents: CHAO, Mark et al.; McDonnell Boehnen Hulbert & Berghoff, 300 South Wacker Drive, Chicago, IL 60606 (US). (81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 13 November 1997 (13.11.97) |
| (54) Title: HUMAN BIKUNIN (57) Abstract <p>The instant invention provides for proteins, polypeptides, nucleic acid sequences, constructs, expression vectors, host cells, pharmaceutical compositions of, and methods for using human placental bikunin, serine protease inhibitor domains, and fragments thereof.</p> | | | |

* (Referred to in PCT Gazette No. 20/1998, Section II)

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|----|--------------------------|----|---------------------------------------|----|--------------------------|
| AM | Armenia | GB | United Kingdom | MW | Malawi |
| AT | Austria | GE | Georgia | MX | Mexico |
| AU | Australia | GN | Guinea | NE | Niger |
| BB | Barbados | GR | Greece | NL | Netherlands |
| BE | Belgium | HU | Hungary | NO | Norway |
| BF | Burkina Faso | IE | Ireland | NZ | New Zealand |
| BG | Bulgaria | IT | Italy | PL | Poland |
| BJ | Benin | JP | Japan | PT | Portugal |
| BR | Brazil | KE | Kenya | RO | Romania |
| BY | Belarus | KG | Kyrgyzstan | RU | Russian Federation |
| CA | Canada | KP | Democratic People's Republic of Korea | SD | Sudan |
| CF | Central African Republic | KR | Republic of Korea | SE | Sweden |
| CG | Congo | KZ | Kazakhstan | SG | Singapore |
| CH | Switzerland | LI | Liechtenstein | SI | Slovenia |
| CI | Côte d'Ivoire | LK | Sri Lanka | SK | Slovakia |
| CM | Cameroon | LR | Liberia | SN | Senegal |
| CN | China | LT | Lithuania | SZ | Swaziland |
| CS | Czechoslovakia | LU | Luxembourg | TD | Chad |
| CZ | Czech Republic | LV | Latvia | TG | Togo |
| DE | Germany | MC | Monaco | TJ | Tajikistan |
| DK | Denmark | MD | Republic of Moldova | TT | Trinidad and Tobago |
| EE | Estonia | MG | Madagascar | UA | Ukraine |
| ES | Spain | ML | Mali | UG | Uganda |
| FI | Finland | MN | Mongolia | US | United States of America |
| FR | France | MR | Mauritania | UZ | Uzbekistan |
| GA | Gabon | | | VN | Viet Nam |

Human Bikunin

Field of the Invention

The compositions of the invention relate to the field of proteins which
5 inhibit serine protease activity. The invention also relates to the field of nucleic acid constructs, vectors and host cells for producing serine protease inhibiting proteins, pharmaceutical compositions containing the protein, and methods for their use.

10 Background of the Invention

Problem Addressed

Blood loss is a serious complication of major surgeries such as open heart surgery and other complicated procedures. Cardiac surgery patients account for a significant proportion of transfused donor blood. Blood transfusion
15 carries risks of disease transmission and adverse reactions. In addition, donor blood is expensive and demands often exceed supply. Pharmacological methods for reducing blood loss and the resultant need for transfusion have been described (reviewed by Scott et al., Ann. Thorac. Surg. 50: 843-851, 1990).

20 *Protein Serine Protease Inhibitor*

Aprotinin, a bovine serine protease inhibitor of the Kunitz family is the active substance in the medicament Trasylol®. Aprotinin (Trasylol®) has been reported as being effective in reducing perioperative blood loss (Royston et al., Lancet ii: 1289-1291, 1987; Dietrich et al., Thorac. Cardiovasc. Surg. 37: 92-98,
25 1989; Fraedrich et al., Thorac. Cardiovasc. Surg. 37: 89-91, 1989); W. van Oeveren et al. (1987), Ann Thorac. Surg. 44, pp 640-645; Bistrup et al., (1988) Lancet I, 366-367), but adverse effects, including hypotension and flushing (Bohrer et al., Anesthesia 45: 853-854, 1990) and allergic reactions (Dietrich et al., Supra) have been reported. Use of aprotinin in patients previously exposed
30 to it is not recommended (Dietrich et al., Supra). Trasylol® has also been used for the treatment of hyperfibrinolytic hemorrhages and traumatic hemorrhagic shock.

Aprotinin is known to inhibit several serine proteases including trypsin, chymotrypsin, plasmin and kallikrein, and is used therapeutically in the
35 treatment of acute pancreatitis, various states of shock syndrome, hyperfibrinolytic hemorrhage and myocardial infarction (Trapnell et al., (1974) Brit J. Surg. 61: 177; J. McMichan et al., (1982) Circulatory Shock 9: 107; Auer et

al., (1979)Acta Neurochir. 49: 207; Sher (1977) Am J. Obstet. Gynecol. 129: 164; Schneider (1976) , Arzneim.-Firsch. 26: 1606). It is generally thought that Trasylol® reduces blood loss *in vivo* through inhibition of kallikrein and plasmin. It has been found that aprotinin (3-58, Arg15, Ala17, Ser42) exhibits
5 improved plasma kallikrein inhibitory potency as compared to native aprotinin itself (WO 89/10374).

Problems With Aprotinin

Because aprotinin is of bovine origin, there is a finite risk of inducing
10 anaphylaxis in human patients upon re-exposure to the drug. Thus, a human functional equivalent to aprotinin, by virtue of a lower risk of anaphylaxis, would be most useful and desirable to have.

Aprotinin is also nephrotoxic in rodents and dogs when administered repeatedly at high dose (Bayer, Trasylol®, Inhibitor of proteinase; Glasser et al.,
15 in "Verhandlungen der Deutschen Gesellschaft fur Innere Medizin, 78. Kongress", Bergmann, Munchen, 1972 pp. 1612-1614). One hypothesis ascribes this effect to the accumulation of aprotinin in the negatively charged proximal tubules of the kidney, due to its high net positive charge (WO 93/14120).

Accordingly, an object of the present invention is to identify human
20 proteins with functional activity similar to aprotinin. It was also an object of the instant invention to identify human proteins, that would be less charged, yet exhibit the same, highly similar, or improved protease specificities as found for aprotinin, especially with respect to the potency of plasmin and kallikrein inhibition. Such inhibitors could then be used repeatedly as medicaments in
25 human patients with reduced risk of adverse immune response and reduced nephrotoxicity.

Brief Summary of the Invention

The instant invention provides for a purified human serine protease
30 inhibitor which can specifically inhibit kallikrein, that has been isolated from human placental tissue via affinity chromatography.

The instant invention provides a newly identified human protein herein called human placental bikunin that contains two serine protease inhibitor domains of the Kunitz class. In one particular embodiment, the instant
35 invention embodies a protein having the amino acid sequence:

5 ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
 YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
 NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
 ACMLRCFRQQ ENPPLPLGSK VVVLGAVS 179
 (SEQ ID NO: 1)

In a preferred embodiment the instant invention provides for native human placental bikunin protein having the amino acid sequence:

10 ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
 YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
 NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
 ACMLRCFRQQ ENPPLPLGSK 170
 (SEQ ID NO: 52)

15 In one aspect, the biological activity of the protein of the instant invention is that it can bind to and substantially inhibit the biological activity of trypsin, human plasma and tissue kallikreins, human plasmin and Factor XIIa. In a preferred embodiment, the present invention provides for a native human placental bikunin protein, in glycosylated form. In a further embodiment the instant invention encompasses native human bikunin protein which has been formed such that it contains at least one cysteine-cysteine disulfide bond. In a preferred embodiment, the protein contains at least one intra-chain cysteine-cysteine disulfide bond formed between a pair of cysteines selected from the group consisting of CYS11-CYS61, CYS20-CYS44, CYS36-CYS57, CYS106-CYS156, CYS115-CYS139, and CYS131-CYS152, wherein the cysteines are numbered according to the amino acid sequence of native human placental bikunin. One of ordinary skill will recognize that the protein of the instant invention may fold into the proper three-dimensional conformation, such that the biological activity of native human bikunin is maintained, where none, one or more, or all of the native intra-chain cysteine-cysteine disulfide bonds are present. In a most preferred embodiment, the protein of the instant invention is properly folded and is formed with all of the proper native cysteine-cysteine disulfide bonds.

35 Active protein of the instant invention can be obtained by purification from human tissue, such as placenta, or via synthetic protein chemistry techniques, as illustrated by the Examples below. It is also understood that the

protein of the instant invention may be obtained using molecular biology techniques, where self-replicating vectors are capable of expressing the protein of the instant invention from transformed cells. Such protein can be made as non-secreted, or secreted forms from transformed cells. In order to facilitate secretion from transformed cells, to enhance the functional stability of the translated protein, or to aid folding of the bikunin protein, certain signal peptide sequences may be added to the NH₂-terminal portion of the native human bikunin protein.

In one embodiment, the instant invention thus provides for the native human bikunin protein with at least a portion of the native signal peptide sequence intact. Thus one embodiment of the invention provides for native human bikunin with at least part of the signal peptide, having the amino acid sequence:

15 AGSFLAWLGSLLLSGVLA -1
 ADRETSIHDFCLVSKVVGRCRASMPRWYNVTDGSCQLFVYGGCDGNSNN 50
 YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDSEDHSSDMF 100
 NYEEYCTANAVTGPCRASFPWYFDVERNSCNNFIYGGCRGNKNSYRSEE 150
 ACMLRCFRQQENPPLPLGSKVVVLGAVS 179
 20 (SEQ ID NO: 2)

In a preferred embodiment the instant invention provides for a native human placental bikunin protein with part of the leader sequence intact, having the amino acid sequence of SEQ ID NO: 52 with an intact leader segment having the amino acid sequence:

25 MAQLCGL RRSRAFLALL GSLLLSGVLA -1
 (SEQ ID NO: 53)

In another embodiment, the instant invention provides for bikunin protein with part of the leader sequence intact, having the amino acid sequence of SEQ ID NO: 52 with the intact leader segment having the amino acid sequence:

30 MLR AEADGVSRL L GSLLLSGVLA -1
 (SEQ ID NO: 54)

35 In a preferred numbering system used herein the amino acid numbered +1 is assigned to the NH₂-terminus of the amino acid sequence for native

human placental bikunin. One will readily recognize that functional protein fragments can be derived from native human placental bikunin, which will maintain at least part of the biological activity of native human placental bikunin, and act as serine protease inhibitors.

5 In one embodiment, the protein of the instant invention comprises a fragment of native human placental bikunin, which contains at least one functional Kunitz-like domain, having the amino acid sequence of native human placental bikunin amino acids 7-159, hereinafter called "bikunin (7-159)". Thus the instant invention embodies a protein having the amino acid
10 sequence:

```

IHDFCLVSKVVGRCRASMPRWYNVTDGSCQLFVYGGCDGNSNN      50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDSEDHSSDMF 100
NYEEYCTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE 150
15 ACMLRCFRQ                                           159
(SEQ ID NO: 3)

```

where the amino acid numbering corresponds to that of the amino acid sequence of native human placental bikunin. Another functional variant of this
20 embodiment can be the fragment of native human placental bikunin, which contains at least one functional Kunitz-like domain, having the amino acid sequence of native human placental bikunin amino acids 11-156, bikunin (11-156)

```

25 CLVSKVVGRCRASMPRWYNVTDGSCQLFVYGGCDGNSNN      50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDSEDHSSDMF 100
NYEEYCTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE 150
ACMLRC                                           156
30 (SEQ ID NO: 50).

```

One can recognize that the individual Kunitz-like domains are also fragments of the native placental bikunin. In particular, the instant invention provides for a protein having the amino acid sequence of a first Kunitz-like domain consisting of the amino acid sequence of native human placental
35 bikunin amino acids 7-64, hereinafter called "bikunin (7-64)". Thus in one embodiment the instant invention encompasses a protein which contains at least one Kunitz-like domain having the amino acid sequence:

IHDFCLVSKVVGRASMPRWYNVTDGSCQLFVYGGCDGNSNN 50
 YLTKEECLKKCATV 64
 (SEQ ID NO: 4)

5

where the amino acid numbering corresponds to that of the amino acid sequence of native human placental bikunin. Another form of the protein of the instant invention can be a first Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 11-61, "bikunin (11-10 61)" having the amino acid sequence:

CLVSKVVGRASMPRWYNVTDGSCQLFVYGGCDGNSNN 50
 YLTKEECLKKC 61
 (SEQ ID NO: 5)

15

The instant invention also provides for a protein having the amino acid sequence of a Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 102-159, hereinafter called "bikunin (102-159)". Thus one embodiment the instant invention encompasses a 20 protein which contains at least one Kunitz-like domain having the amino acid sequence:

YEEYCTANAVTGPCRASFPWYFDVERNSCNNFIYGGCRGNKNSYRSEE 150
 ACMLRCFRQ 159
 25 (SEQ ID NO: 6)

where the amino acid numbering corresponds to that of the amino acid sequence of native human placental bikunin. Another form of this domain can be a Kunitz-like domain consisting of the amino acid sequence of native human 30 placental bikunin amino acids 106-156, "bikunin (106-156)" having the amino acid sequence:

CTANAVTGPCRASFPWYFDVERNSCNNFIYGGCRGNKNSYRSEE 150
 ACMLRC 156
 35 (SEQ ID NO: 7)

Thus one of ordinary skill will recognize that fragments of the native

human bikunin protein can be made which will retain at least some of the native protein biological activity. Such fragments can also be combined in different orientations or multiple combinations to provide for alternative proteins which retain some of, the same, or more biological activity of the native human bikunin protein.

One will readily recognize that biologically active protein of the instant invention may comprise one or more of the instant Kunitz-like domains in combination with additional Kunitz-like domains from other sources. Biologically active protein of the instant invention may comprise one or more of the instant Kunitz-like domains in combination with additional protein domains from other sources with a variety of biological activities. The biological activity of the protein of the instant invention can be combined with that of other known protein or proteins to provide for multifunctional fusion proteins having predictable biological activity. Thus, in one embodiment, the instant invention encompasses a protein which contains at least one amino acid sequence segment the same as, or functionally equivalent to the amino acid sequence of either SEQ ID NO: 5 or SEQ ID NO: 7.

An open reading frame which terminates at an early stop codon can still code for a functional protein. The instant invention encompasses such alternative termination, and in one embodiment provides for a protein of the amino acid sequence:

```

ADRERSIHDFCLVSKVVGRCPASMPRWYNYVTDGSCQLFVYGGCDGNSNN      50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDS                92
(SEQIDNO:8)

```

In one embodiment, the instant invention provides for substantially purified, or recombinantly produced native human bikunin protein with an intact segment of the leader sequence, and at least a portion of the native transmembrane region intact. Thus one embodiment of the invention provides for native human bikunin, with an intact leader sequence, and with at least part of the transmembrane domain (underlined), having an amino acid sequence selected from:

| | | | | |
|----|-------------------|-----------------------|-----------------------|-----|
| | 1) EST | MLR AEADGVSRL | GSLLLSGVLA | -1 |
| | 2) PCR | MAQLCGL RRSRAFLALL | GSLLLSGVLA | -1 |
| | 3) λ cDNA | MAQLCGL RRSRAFLALL | GSLLLSGVLA | -1 |
| 5 | 1) ADRERSIHDF | CLVSKVVGRC RASMPRWYN | VTDGSCQLFV YGGCDGNSNN | 50 |
| | 2) ADRERSIHDF | CLVSKVVGRC RASMPRWYN | VTDGSCQLFV YGGCDGNSNN | 50 |
| | 3) ADRERSIHDF | CLVSKVVGRC RASMPRWYN | VTDGSCQLFV YGGCDGNSNN | 50 |
| 10 | 1) YLTKEECLFK | CATVTENATG DLATSRNAAD | SSVPSAPRRQ DSEDHSSDMF | 100 |
| | 2) YLTKEECLFK | CATVTENATG DLATSRNAAD | SSVPSAPRRQ DSEDHSSDMF | 100 |
| | 3) YLTKEECLFK | CATVTENATG DLATSRNAAD | SSVPSAPRRQ DSEDHSSDMF | 100 |
| | 1) NYEEYCTANA | VTGPCRASFP RWYFDVERNS | CNNFIYGGCR GNKNSYRSEE | 150 |
| 15 | 2) NYEEYCTANA | VTGPCRASFP RWYFDVERNS | CNNFIYGGCR GNKNSYRSEE | 150 |
| | 3) NYEEYCTANA | VTGPCRASFP RWYFDVERNS | CNNFIYGGCR GNKNSYRSEE | 150 |
| | 1) ACMLRCFRQQ | ENPPLPLGSK VVVLAGLFVM | VLILFLGASM VYLIRVARRN | 200 |
| | 2) ACMLRCFRQQ | ENPPLPLGSK VVVLAGLFVM | VLILFLGASM VYLIRVARRN | 200 |
| 20 | 3) ACMLRCFRQQ | ENPPLPLGSK VVVLAGLFVM | VLILFLGASM VYLIRVARRN | 200 |
| | 1) QERALRTVWS | SGDDKEQLVK NTYVL | | 225 |
| | 2) QERALRTVWS | FGD | | 213 |
| | 3) QERALRTVWS | SGDDKEQLVK NTYVL | | 225 |

- 25 where sequence 1) is EST derived consensus SEQ ID NO: 45, 2) is PCR clone SEQ ID NO: 47, and 3) is lambda cDNA clone SEQ ID NO: 49. In a preferred embodiment a protein of the instant invention comprises one of the amino acid sequence of SEQ ID NO: 45, 47 or 49 wherein the protein has been cleaved in the region between the end of the last Kunitz domain and the transmembrane region.
- 30

The instant invention also embodies the protein wherein the signal peptide is deleted. Thus the instant invention provides for a protein having the amino acid sequence of SEQ ID NO: 52 continuous with a transmembrane amino acid sequence:

| | | | |
|----|-----|----------------------------------|-----|
| 35 | EST | VVVLAGLFVM VLILFLGASM VYLIRVARRN | 200 |
| | EST | QERALRTVWS SGDDKEQLVK NTYVL | 225 |
| | | (SEQ ID NO: 69) | |

a transmembrane amino acid sequence:

| | | | |
|----|-----|----------------------------------|-----|
| | PCR | VVVLAGLFVM VLILFLGASM VYLIRVARRN | 200 |
| 40 | PCR | QERALRTVWS FGD | 213 |
| | | (SEQ ID NO: 68) | |

or a transmembrane amino acid sequence:

| | | | |
|----|----------------|----------------------------------|-----|
| | λ cDNA | VVVLAGLFVM VLILFLGASM VYLIRVARRN | 200 |
| | λ cDNA | QERALRTVWS SGDDKEQLVK NTYVL | 225 |
| 45 | | (SEQ ID NO: 67) | |

The protein amino acid sequences of the instant invention clearly teach one of the art the appropriate nucleic acid sequences which can be used in molecular biology techniques to produce the proteins of the instant invention. Thus, one embodiment of the instant invention provides for a nucleic acid sequence which encodes for a human bikunin having the consensus DNA sequence of Figure 3 (SEQ ID NO: 9), which translates into the amino acid sequence for native human placental bikunin sequence of Figure 3 (SEQ ID NO: 10). In another embodiment, the instant invention provides for a consensus nucleic acid sequence of Figure 4C (SEQ ID NO: 51) which encodes for an amino acid sequence of Figure 4D (SEQ ID NO: 45).

In a preferred embodiment, the instant invention provides for a nucleic acid sequence which encodes for native human placental bikunin having the DNA sequence of Figure 4F (SEQ ID NO: 48) which encodes for the protein sequence of SEQ ID NO: 49. In another embodiment, the instant invention provides for a nucleic acid sequence of Figure 4E (SEQ ID NO: 46) which encodes for a protein sequence of SEQ ID NO: 47.

One can easily recognize that certain allelic mutations, and conservative substitutions made in the nucleic acid sequence can be made which will still result in a protein amino acid sequence encompassed by the instant invention. One of skill in the art can recognize that certain natural allelic mutations of the protein of the instant invention, and conservative substitutions of amino acids in the protein of the instant invention will not significantly alter the biological activity of the protein, and are encompassed by the instant invention.

The instant invention also provides for pharmaceutical compositions containing human placental bikunin and fragments thereof that are useful for the reduction of perioperative blood loss in a patient undergoing surgery.

The present invention also provides methods for reducing perioperative blood loss in a patient undergoing surgery, wherein an effective amount of the disclosed human serine protease inhibitors of the present invention in a biologically compatible vehicle is administered to the patient.

The present invention also provides for variants of placental bikunin, and the specific Kunitz domains described above, that contain amino acid substitutions that alter the protease specificity. Preferred sites of substitution are indicated below as positions Xaa¹ through Xaa³² in the amino acid sequence for native placental bikunin. Substitutions at Xaa¹ through Xaa¹⁶ are also preferred for variants of bikunin (7-64), while substitutions at Xaa¹⁷ through Xaa³² are preferred for variants of bikunin (102-159).

Thus the present invention embodies protein having an amino acid sequence:

| | | |
|----|---|-----|
| | Ala Asp Arg Glu Arg Ser Ile Xaa ¹ Asp Phe | 10 |
| | Cys Leu Val Ser Lys Val Xaa ² Gly Xaa ³ Cys | 20 |
| 5 | Xaa ⁴ Xaa ⁵ Xaa ⁶ Xaa ⁷ Xaa ⁸ Xaa ⁹ Trp Trp Tyr Asn | 30 |
| | Val Thr Asp Gly Ser Cys Gln Leu Phe Xaa ¹⁰ | 40 |
| | Tyr Xaa ¹¹ Gly Cys Xaa ¹² Xaa ¹³ Xaa ¹⁴ Ser Asn Asn | 50 |
| | Tyr Xaa ¹⁵ Thr Lys Glu Glu Cys Leu Lys Lys | 60 |
| | Cys Ala Thr Xaa ¹⁶ Thr Glu Asn Ala Thr Gly | 70 |
| 10 | Asp Leu Ser Thr Ser Arg Asn Ala Ala Asp | 80 |
| | Ser Ser Val Pro Ser Ala Pro Arg Arg Gln | 90 |
| | Asp Ser Glu His Asp Ser Ser Asp Met Phe | 100 |
| | Asn Tyr Xaa ¹⁷ Glu Tyr Cys Thr Ala Asn Ala | 110 |
| | Val Xaa ¹⁸ Gly Xaa ¹⁹ Cys Xaa ²⁰ Xaa ²¹ Xaa ²² Xaa ²³ Xaa ²⁴ | 120 |
| 15 | Xaa ²⁵ Trp Tyr Phe Asp Val Glu Arg Asn Ser | 130 |
| | Cys Asn Asn Phe Xaa ²⁶ Tyr Xaa ²⁷ Gly Cys Xaa ²⁸ | 140 |
| | Xaa ²⁹ Xaa ³⁰ Lys Asn Ser Tyr Xaa ³¹ Ser Glu Glu | 150 |
| | Ala Cys Met Leu Arg Cys Phe Arg Xaa ³² Gln | 160 |
| | Glu Asn Pro Pro Leu Pro Leu Gly Ser Lys | 170 |
| 20 | Val Val Val Leu Ala Gly Ala Val Ser | 179 |
| | (SEQ ID NO: 11). | |

where Xaa¹ - Xaa³² each independently represents a naturally occurring amino acid residue except Cys, with the proviso that at least one of the amino acid residues Xaa¹-Xaa³² is different from the corresponding amino acid residue of the native sequence.

In the present context, the term "naturally occurring amino acid residue" is intended to indicate any one of the 20 commonly occurring amino acids, i.e., Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.

By substituting one or more amino acids in one or more of the positions indicated above, it may be possible to change the inhibitor specificity profile of native placental bikunin or that of the individual Kunitz-like domains, bikunin(7-64) or bikunin (102-159) so that it preferentially inhibits other serine proteases such as, but not limited to, the enzymes of the complement cascade, TF/FVIIa, FXa, thrombin, neutrophil elastase, cathepsin G or proteinase-3.

Examples of preferred variants of placental bikunin include those

wherein Xaa¹ is an amino acid residue selected from the group consisting of His, Glu, Pro, Ala, Val or Lys, in particular wherein Xaa¹ is His or Pro; or wherein Xaa² is an amino acid residue selected from the group consisting of Val, Thr, Asp, Pro, Arg, Tyr, Glu, Ala, Lys, in particular wherein Xaa² is Val or Thr; or wherein Xaa³ is an amino acid residue selected from the group consisting of Arg, Pro, Ile, Leu, Thr, in particular wherein Xaa³ is Arg or Pro; or wherein Xaa⁴ is an amino acid residue selected from the group consisting of Arg, Lys and Ser, Gln, in particular wherein Xaa⁴ is Arg or Lys; or wherein Xaa⁵ is an amino acid residue selected from the group consisting of Ala, Gly, Asp, Thr, in particular wherein Xaa⁵ is Ala; or wherein Xaa⁶ is an amino acid residue selected from the group consisting of Ser, Ile, Tyr, Asn, Leu, Val, Arg, Phe, in particular wherein Xaa⁶ is Ser or Arg; or wherein Xaa⁷ is an amino acid residue selected from the group consisting of Met, Phe, Ile, Glu, Leu, Thr and Val, in particular wherein Xaa⁷ is Met or Ile; or wherein Xaa⁸ is an amino acid residue selected from the group consisting of Pro, Lys, Thr, Gln, Asn, Leu, Ser or Ile, in particular wherein Xaa⁸ is Pro or Ile; or wherein Xaa⁹ is an amino acid residue selected from the group consisting of Arg, Lys or Leu, in particular wherein Xaa⁹ is Arg; or wherein Xaa¹⁰ is an amino acid residue selected from the group consisting of Val, Ile, Lys, Ala, Pro, Phe, Trp, Gln, Leu and Thr, in particular wherein Xaa¹⁰ is Val; or wherein Xaa¹¹ is an amino acid residue selected from the group consisting of Gly, Ser and Thr, in particular wherein Xaa¹¹ is Gly; or wherein Xaa¹² is an amino acid residue selected from the group consisting of Asp, Arg, Glu, Leu, Gln, Gly, in particular wherein Xaa¹² is Asp or Arg; or wherein Xaa¹³ is an amino acid residue selected from the group consisting of Gly and Ala; or wherein Xaa¹⁴ is an amino acid residue selected from the group consisting of Asn or Lys; or wherein Xaa¹⁵ is an amino acid residue selected from the group consisting of Gly, Asp, Leu, Arg, Glu, Thr, Tyr, Val, and Lys, in particular wherein Xaa¹⁵ is Leu or Lys; or wherein Xaa¹⁶ is an amino acid residue selected from the group consisting of Val, Gln, Asp, Gly, Ile, Ala, Met, and Val, in particular wherein Xaa¹⁶ is Val or Ala; or wherein Xaa¹⁷ is an amino acid residue selected from the group consisting of His, Glu, Pro, Ala, Lys and Val, in particular wherein Xaa¹⁷ is Glu or Pro; or wherein Xaa¹⁸ is an amino acid residue selected from the group consisting of Val, Thr, Asp, Pro, Arg, Tyr, Glu, Ala or Lys, in particular wherein Xaa¹⁸ is Thr; or wherein Xaa¹⁹ is an amino acid residue selected from the group consisting of Arg, Pro, Ile, Leu or Thr, in particular wherein Xaa¹⁹ is Pro; or wherein Xaa²⁰ is an amino acid residue selected from the group consisting of Arg, Lys, Gln and

Ser, in particular wherein Xaa²⁰ is Arg or Lys; or wherein Xaa²¹ is an amino acid residue selected from the group consisting of Ala, Asp, Thr or Gly ; in particular wherein Xaa²¹ is Ala; or wherein Xaa²² is an amino acid residue selected from the group consisting of Ser, Ile, Tyr, Asn, Leu, Val, Arg or Phe, in particular wherein Xaa²² is Ser or Arg ; or wherein Xaa²³ is an amino acid residue selected from the group consisting of Met, Phe, Ile, Glu, Leu, Thr and Val, in particular wherein Xaa²³ is Phe or Ile; or wherein Xaa²⁴ is an amino acid residue selected from the group consisting of Pro, Lys, Thr, Asn, Leu, Gln, Ser or Ile, in particular wherein Xaa²⁴ is Pro or Ile; or wherein Xaa²⁵ is an amino acid residue selected from the group consisting of Arg, Lys or Leu, in particular wherein Xaa²⁵ is Arg; or wherein Xaa²⁶ is an amino acid residue selected from the group consisting of Val, Ile, Lys, Leu, Ala, Pro, Phe, Gln, Trp and Thr, in particular wherein Xaa²⁶ is Val or Ile; or wherein Xaa²⁷ is an amino acid residue selected from the group consisting of Gly, Ser and Thr, in particular wherein Xaa²⁷ is Gly; or wherein Xaa²⁸ is an amino acid residue selected from the group consisting of Asp, Arg, Glu, Leu, Gly or Gln, in particular wherein Xaa²⁸ is Arg; or wherein Xaa²⁹ is an amino acid residue selected from the group consisting of Gly and Ala; or wherein Xaa³⁰ is an amino acid residue selected from the group consisting of Asn or Lys; or wherein Xaa³¹ is an amino acid residue selected from the group consisting of Gly, Asp, Leu, Arg, Glu, Thr, Tyr, Val, and Lys, in particular wherein Xaa³¹ is Arg or Lys; or wherein Xaa³² is an amino acid residue selected from the group consisting of Val, Gln, Asp, Gly, Ile, Ala, Met, and Thr, in particular wherein Xaa³² is Gln or Ala.

Description of the Drawings

The invention will be better understood from a consideration of the following detailed description and claims, taken in conjunction with the drawings, in which:

Figure 1 depicts the nucleotide sequence of EST R35464 (SEQ ID NO: 12) and the translation of this DNA sequence (SEQ ID NO: 13) which yielded an open reading frame with some sequence similarity to aprotinin. The translation product contains 5 of the 6 cysteines in the correct spacing that is characteristic for Kunitz-like inhibitor domains (indicated in bold). The position normally occupied by the remaining cysteine (at codon 38) contained instead a phenylalanine (indicated by an asterisk).

Figure 2 depicts the nucleotide sequence of EST R74593 (SEQ ID NO: 14),

and the translation of this DNA sequence (SEQ ID NO: 15) which yielded an open reading frame with homology to the Kunitz class of serine protease inhibitor domains. The translation product contained 6 cysteines in the correct spacing that is characteristic for Kunitz-like inhibitor domains (indicated in bold). However, this reading frame sequence includes stop codons at codon 3 and 23.

Figure 3 depicts a deduced nucleic acid sequence of human placental bikunin (SEQ ID NO: 9) labeled "consensus" and matched with the translated protein amino acid sequence labeled "translated" (SEQ ID NO: 10). Also as comparison are shown the nucleic acid sequence for ESTs H94519 (SEQ ID NO: 16), N39798 (SEQ ID NO: 17), R74593 (SEQ ID NO: 14) and R35464 (SEQ ID NO: 12). The underlined nucleotides in the consensus sequence correspond to the site of PCR primers described in the Examples. Underlined amino acids in the translated consensus sequence are residues whose identity have been confirmed by amino acid sequencing of purified native human placental bikunin. Nucleotide and amino acid code are standard single letter code, "N" in the nucleic acid code indicates an unassigned nucleic acid, and "*" indicates a stop codon in the amino acid sequence.

Figure 4A depicts the original overlay of a series of ESTs with some nucleic acid sequence homology to ESTs encoding human placental bikunin, or portions thereof. Shown for reference are the relative positions of bikunin (7-64) and bikunin (102-159), labeled KID1 and KID2 respectively.

Figure 4B depicts a subsequent more comprehensive EST overlay incorporating additional ESTs. Numbers on the upper X-axis refer to length in base pairs, starting at the first base from the most 5' EST sequence. The length of each bar is in proportion to the length in base pairs of the individual ESTs including gaps. The EST accession numbers are indicated to the right of their respective EST bars.

Figure 4C depicts the corresponding alignment of the oligonucleotide sequences of each of the overlapping ESTs shown schematically in Figure 4B. The upper sequence (SEQ ID NO: 51) labeled bikunin represents the consensus oligonucleotide sequence derived from the overlapping nucleotides at each position. The numbers refer to base-pair position within the EST map. The oligonucleotides in EST R74593 that are bold underlined (at map positions 994 and 1005) are base insertions observed in R74593 that were consistently absent in each of the other overlapping ESTs.

Figure 4D depicts the amino acid translation of the consensus

oligonucleotide sequence for bikunin depicted in Figure 4C (SEQ ID NO: 45).

Figure 4E depicts the nucleotide sequence (SEQ ID NO: 46) and corresponding amino acid translation (SEQ ID NO: 47) of a placental bikunin encoding sequence that was derived from a human placental cDNA library by PCR-based amplification.

Figure 4F depicts the nucleotide sequence (SEQ ID NO: 48) and corresponding amino acid translation (SEQ ID NO: 49) of a native human placental bikunin encoding clone that was isolated from a human placental lambda cDNA library by colony hybridization.

Figure 4G compares the alignment of the amino acid translated oligonucleotide sequences for placental bikunin obtained by EST overlay (SEQ ID NO: 45), PCR based cloning (SEQ ID NO: 47), and conventional lambda colony hybridization (SEQ ID NO: 49).

Figure 5 shows a graph of purification of human placental bikunin from placental tissue after Superdex 75 Gel-Filtration. The plot is an overlay of the protein elution profile as measured by OD 280 nm (solid line), activity of eluted protein in a trypsin inhibition assay (% inhibition shown by circles), and activity of eluted protein in a kallikrein inhibition assay (% inhibition shown by squares).

Figure 6 shows a graph which plots the purification of human placental bikunin from placental tissue using C18 Reverse-Phase Chromatography. The plot is an overlay of the protein elution profile as measured by OD 215 nm (solid line), activity of eluted protein in a trypsin inhibition assay (% inhibition shown by circles), and activity of eluted protein in a kallikrein inhibition assay (% inhibition shown by squares).

Figure 7 depicts a silver stained SDS-PAGE gel of highly purified placental bikunin (lane 2), and a series of molecular size marker proteins (lane 1) of the indicated sizes in kilodaltons. Migration was from top to bottom.

Figure 8 shows the amount of trypsin inhibitory activity present in the cell-free fermentation broth from the growth of yeast strains SC101 (panel 8A) or WHL341 (panel 8B) that were stably transformed with a plasmid (pS604) that directs the expression of placental bikunin (102-159).

Figure 9 shows both a silver stained SDS-PAGE (left panel) and a Western blot with anti-placental bikunin (102-159) pAb (right panel) of cell-free fermentation broth from the growth of yeast strain SC101 (recombinants 2.4 and 2.5) that was stably transformed with a plasmid directing the expression of either bovine aprotinin, or placental bikunin (102-159). Migration was from top

to bottom.

Figure 10 is a photograph which shows a silver stained SDS-PAGE of highly purified placental bikunin (102-159) (lane 2) and a series of molecular size marker proteins (lane 1) of the indicated sizes in Kilodaltons. Migration was from top to bottom.

Figure 11 is a photograph which shows the results of Northern blots of mRNA from various human tissues that was hybridized to a ^{32}P labeled cDNA probe encoding either placental bikunin (102-159) (panel 11A) or encoding placental bikunin (1-213) (panel 11B). Migration was from top to bottom. The numbers to the right of each blot refer to the size in kilobases of the adjacent RNA markers. The organs from which mRNA was derived is described under each lane of the blot.

Figure 12 depicts an immunoblot of placental derived placental bikunin with rabbit antiserum raised against either synthetic reduced placental bikunin (7-64) (panel A) or 102-159 (panel B). For each panel, contents were: molecular size markers (lanes 1); native placental bikunin isolated from human placenta (lanes 2); synthetic placental bikunin (7-64) (lanes 3) and synthetic placental bikunin (102-159) (lanes 4). Tricine 10-20% SDS-PAGE gels were blotted and developed with protein A-purified primary polyclonal antibody (8 ug IgG in 20 ml 0.1% BSA/Tris-buffered saline (pH 7.5), followed by alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. Migration was from top to bottom.

Figure 13 depicts a Coomassie Blue stained 10-20% Tricine SDS-PAGE gel of 3 micrograms of highly purified placental bikunin (1-170) derived from a baculovirus / Sf9 expression system (lane 2). Lane 1 contains molecular size markers. Migration was from top to bottom.

Figure 14 depicts a comparison of the effect of increasing concentrations of either Sf9-derived human placental bikunin (1-170) (filled circles), synthetic placental bikunin (102-159) (open circles), or aprotinin (open squares) on the activated partial thromboplastin time of human plasma. Clotting was initiated with CaCl_2 . The concentration of proteins are plotted versus the -fold prolongation in clotting time. The uninhibited clotting time was 30.8 seconds.

Detailed Description of the Invention

The present invention encompasses a newly identified human protein herein called human placental bikunin that contains two serine protease inhibitor domains of the Kunitz class. The instant invention also encompasses

pharmaceutical compositions containing placental bikunin and fragments thereof that are useful for the reduction of perioperative blood loss in a patient undergoing surgery, or with major trauma.

5 The present invention also provides methods for reducing perioperative blood loss in a patient undergoing surgery or due to major trauma, wherein an effective amount of the disclosed human serine protease inhibitors of the present invention, in a biologically compatible vehicle, is administered to the patient.

10 A preferred application for placental bikunin, isolated domains, and other variants is for the reduction of blood loss resulting from trauma or surgery that has the potential for loss of large volumes of blood. These methods and compositions reduce or eliminate the need for whole donor blood or blood products, thereby reducing the risk of infection and other adverse side effects, as well as the cost of surgery. The methods are thus useful in reducing
15 blood loss in normal patients, i.e., those not suffering from inborn or other pre-operative deficiencies in coagulation factors. The reduction in blood loss is seen as a reduction in blood loss during surgery, as reduced post surgical drainage or both. Preferred surgical applications include but are not limited to use in thoracic and abdominal surgery, total and partial hip replacement surgeries and surgeries to treat a patient having an epithelial lesion of the eye. Preferred
20 thoracic surgical procedures include but are not limited to aortocoronary bypass, excision of cardiac and aortic aneurysms, and surgery for esophageal varices, and coronary artery bypass surgery. Preferred abdominal surgeries include but are not limited to liver transplants, radical prostatectomy, surgery for diverticulitis of colon, tumor debulking, surgery on the abdominal aorta and
25 surgery for duodenal ulcers, and repair of liver or spleen trauma. Preferred use for the treatment of trauma include but are not limited to the use in stabilization of severely injured patients at accident sites suffering from e.g., limb loss or major thoracic /abdominal wounds. In case of use for the
30 reduction of blood loss resulting from surgery it is preferred to administer the placental bikunin, isolated domains, or other variant prior to and during surgery, whereas in case of use in trauma settings the placental bikunin variant, isolated domain or other variant is to be administered as rapidly as possible following injury, and should be contained on emergency vehicles traveling to
35 the accident sites.

Factor XII (also known as Hageman Factor) is a serine protease that is found in the circulation in a zymogen form (80 kD) at approximately 29-40

µg/ml (see Pixley, et al. (1993) *Meth. in Enz.*, 222, 51-64) and is activated by tissue and plasma kallikrein. Once activated, it participates in the intrinsic pathway of blood coagulation which is activated when blood or plasma contacts a "foreign" or anionic surface. Once activated, Factor XIIa can then
5 cleave and activate a number of other plasma proteases including Factor XI, prekallikrein, and C1 of the complement system. Thus Factor XII may be involved in causing hypotensive reactions since activated kallikrein can cleave kininogen releasing bradykinin (see Colman, (1984) *J. Clin. Invest.*, 73, 1249).

Sepsis is a disease that results from bacterial infection due to bacterial
10 endotoxin or lipopolysaccharide (LPS). Exposure of Factor XII to LPS results in the activation of Factor XII. Patients with sepsis frequently have symptoms of intravascular coagulation which may also be due to activation of Factor XII by LPS. Septic shock can result from bacterial infection and is associated with fever, low systemic vascular resistance, and low arterial pressure. It is a
15 common cause of death in intensive care units in the United States, where seventy five percent of the patients that die from septic shock have a persistent hypotension (see Parillo, et al. (1989) *Ann Rev. Med.*, 40, 469-485).

Adult respiratory distress syndrome is characterized by pulmonary edema, hypoxemia, and decreased pulmonary compliance. The pathogenesis of
20 the disease is currently unknown although the proteolytic pathways of coagulation and fibrinolysis are believed to play a role (see Carvalho, et al. (1988) *J. Lab Clin. Med.*, 112: 270-277).

The proteins of the instant invention are also a novel human Kunitz type inhibitor of kallikrein, an activator of Factor XII. Thus another object of the
25 current invention is to present a method for the prophylactic or therapeutic treatment of systemic inflammatory reactions such as septic shock, adult respiratory distress syndrome (ARDS), preeclampsia, multiple organ failure and disseminated intravascular coagulation (DIC). The therapeutic or prophylactic administration of the peptides of the instant invention would
30 result in the modulation of these inflammatory conditions and be beneficial to the patient.

Plasmin plays an important role in extracellular matrix degradation and the activation of matrix-metallo protease (MMP) cascades. Collectively these
35 proteases mediate migration of and tissue invasion by both endothelial cells during angiogenesis/neovascularization, and cancer cells during metastasis. Neovascularization is essential to support tumor growth and metastasis is a process which mediates the spreading of tumors and which is associated with

extremely poor patient prognosis.

Several preclinical studies suggest that Kunitz like serine protease inhibitors with a protease specificity similar to aprotinin are useful as medicaments for cancer. For example, aprotinin reduced tumor growth and invasion, with increased tumor necrosis when administered to hamsters bearing a highly invasive fibrosarcoma or to mice bearing a similarly malignant mammary carcinoma (Latner et al., (1974), Br. J. Cancer 30: 60-67; Latner and Turner, (1976), Br. J. Cancer 33: 535-538). Furthermore, administration of 200,000 KIU of aprotinin i.p. to C57B1/6 Cr male mice on days 1 to 14 post-inoculation with Lewis lung carcinoma cells, reduced pulmonary metastases by 50% although had no effect on primary tumor mass (Giraldi et al., (1977) Eur. J. Cancer, 13: 1321-1323). Similarly, administration of 10,000 KIU i.p. on each of days 13-16 post-inoculation of C57BL/6J mice with Lewis tumor cells inhibited pulmonary metastases by 90% without affecting the primary tumor growth (Uetsuji et al., (1992), Jpn. J. Surg. 22: 429-442). In this same study, administration of plasmin or kallikrein with the same dosing schedule was argued to increase the number of pulmonary metastases. These results prompted the authors to suggest that perioperative administration of aprotinin to cancer patients may reduce the likelihood of metastases. Black and Steger (1976, Eur. J. Pharmacol., 38: 313-319) found that aprotinin inhibited the growth of the transplanted rodent Murphy-Strum lymphosarcoma in rats and suggested that the effect involved the inhibition of the kinin-forming enzyme system. Twice daily i.p. injection of female ddY mice with 10,000 KIU of aprotinin for 7 weeks to mice each bearing a single autochthonous squamous cell carcinoma resulting from 3-methylcholanthrene treatment reduced the growth rate of the primary tumors by 90%. In some animals tumor regression was observed. While all vehicle treated animals had died within the seven weeks, all of the aprotinin treatment group remained alive. Reduced tumor growth was associated with hyperkeratosis (Ohkoshi, Gann (1980), 71: 246-250).

Clinically, a surgically cured group of 26 patients who received aprotinin i.v. exhibited a 70% survival two years post surgery with no recurrence of tumors whereas a placebo group of 26 patients at the same time exhibited only a 38% survival with a significant rate of tumor recurrence (Freeman et al. Br. Soc. Gastroenterol. (1980) supplement A: 902). In a case study (Guthrie et al., Br. J. Clin. Pract (1981) 35: 330-332), administration of bromocriptine plus aprotinin to a patient with advanced cancer of the cervix caused remission. Aprotinin was administered both as a 500,000 KIU i.p. bolus every eight hours concurrently

with a continuous i.v. infusion of aprotinin at a rate of 200,000 KIU per 6 hr for a total of seven days once a month. Treatment was ended at the end of the fourth month due to the development of an allergic reaction to aprotinin. More recent evidence has further underscored a role of plasmin as a target for these effects of aprotinin on metastases.

The mechanism for these events could be related to the fact that aprotinin blocks the invasive potential of cancer cell lines (Liu G., et al., Int J. Cancer (1995), 60: 501-506). Furthermore, since the proteins of the instant invention are also potent inhibitors of plasmin and kallikrein, they are contemplated for use as anti-cancer agents. For example they are contemplated for use in blocking primary tumor growth by restricting neovascularization, primary tumor invasion and in blocking metastasis through inhibition of tissue infiltration. The compounds may be administered locally to tumors or systemically. In a preferred mode of treatment, the protein would be administered perioperatively during tumor debulking to minimize the risk of metastasis. In such a regime, the blood sparing properties of the compound would be additionally advantageous in providing a clearer surgical field of view. Another preferred mode of administration would be as a combination therapy with either MMP inhibitors or chemotherapy. An additional preferred mode of administration would be as a locally administered gene therapy designed to achieve selective expression of placental bikunin within the tumor cells, or their associated stroma and vascular beds.

Preferred types of cancers targeted for therapy would be vascular-dependent solid tumors such as breast, colon, lung, prostate and ovarian carcinomas which exhibit a high metastatic potential, and those for which local delivery of a high concentration of the protein is feasible such as lung cancers through pulmonary delivery, colon carcinomas through hepatic delivery to liver metastasis, or skin cancers such as head and neck carcinomas or melanomas through subcutaneous delivery. Since the proteins of the present invention are of human origin they would be less likely to be associated with allergic or anaphylactic reactions of the kind observed by Guthrie et al., *supra*, upon reuse.

Additionally, the proteins of the present invention are contemplated for use in the reduction of thromboembolic complications associated with activation of the intrinsic pathway of coagulation. This would include prevention of pulmonary embolism in late stage cancer patients, a frequent cause of death (Donati MB., (1994), Haemostasis 24: 128-131).

Edema of the brain and spinal cord is a complication resulting from traumatic brain or spinal cord injury, stroke, cerebral ischemia, cerebral and sub-arachnoid hemorrhage, surgery (including open heart surgery), infectious diseases such as encephalitis and meningitis, granulomatous diseases such as Sarcoid and focal or diffuse carcinomas, and is a contributor to the high level of morbidity and death following these events. Bradykinin is known to disrupt the blood brain barrier experimentally (Greenwood J., (1991), *Neuroradiology*, 33: 95-100; Whittle et al., (1992), *Acta Neurochir.*, 115: 53-59), and infusion of bradykinin into the internal carotid artery induced brain edema in spontaneously hypertensive rats (SHR) subjected to common carotid artery occlusion (Kamiya, (1990), *Nippon Ika Daigaku Zasshi.* 57: 180-191). Elevated levels of bradykinin are found in extracellular fluids following trauma in a model involving traumatized rat spinal chord (Xu et al., (1991), *J. Neurochem.*, 57: 975-980), and in plasma and tissue from rats with brain edema resulting from cerebral ischaemia (Kamiya et al., (1993), *Stroke*, 24: 571-575). Bradykinin is released from high molecular weight kininogen by serine proteases including kallikrein (Coleman (1984) *J. Clin Invest.*, 73: 1249), and the serine protease inhibitor aprotinin was found to block the magnitude of brain edema resulting from cerebralschemia in SHR rats (Kamiya, (1990), *Nippon Ika Daigaku Zasshi.* 57: 180-191; Kamiya et al., (1993), *Stroke*, 24: 571-575) and rabbits subjected to a cold lesion of the brain (Unterberg et al., (1986), *J. Neurosurgery*, 64: 269-276).

These observations indicate that brain edema results from local proteolytic release of kinins such as bradykinin from high molecular weight kininogen, followed by bradykinin-induced increases in blood brain barrier permeability. Accordingly, placental bikunin and fragments thereof are contemplated as medicaments for the prevention of edema in patients at risk for this condition, particularly those of high risk of mortality or brain injury. This would include head and spinal trauma patients, polytrauma patients, patients undergoing surgery of the brain or spinal cord and their associated vessels or other general surgeries including open-heart surgery, patients who have suffered from a stroke, cerebral or sub-arachnoid hemorrhage, infectious diseases of the brain, granulomatous disease of the brain or diffuse or focal carcinomas and tumors of the brain or any conditions such as multiple sclerosis involving breakdown of the blood brain barrier or patients suffering from any other inflammatory processes of the brain or spinal cord. Patients would receive an administration of placental bikunin either as an infusion or bolus injection, intravenously or intracranially. Additional doses of placental bikunin

could be administered intermittently over the following one to three weeks. Dose levels would be designed to attain circulating concentrations in excess of those required to neutralize elevations in plasma levels of bradykinin and other vasoactive peptides formed through the action of serine proteases, and sufficient to reduce edema. Since the protein is of human origin, repeated administration in this course of therapy would not lead to development of an immune reaction to the protein. Placental bikunin and fragments thereof would be contemplated for monotherapy or prophylaxis as well as for use in combination with other medicaments such as neurotherapeutics and neuroprotectants.

Recent evidence (Dela Cadena R. A. et al., (1995), FASEB J. 9: 446-452) has indicated that the contact activation pathway may contribute to the pathogenesis of arthritis and anemia, and that kallikrein inhibitors may be of therapeutic benefit. Accordingly, protease inhibitors of the present invention are contemplated according to their capacity to inhibit human kallikrein, as medicaments for the treatment of arthritis and anemia in humans.

Treatment of male non-insulin diabetic (NIDDM) patients with aprotinin significantly improved total glucose uptake and decreased the metabolic clearance rate of insulin (Laurenti et al., (1996), Diabetic Medicine 13: 642-645). Accordingly, the human proteins of the present invention are contemplated for chronic use as medicaments for the treatment of NIDDM.

Daily treatment of patients at risk of preterm delivery with urinary trypsin inhibitor for two weeks significantly reduced recurrent uterine contractions (Kanayama et al., (1996), Eur J. Obstet. Gynecol. & Reprod. Biol. 67: 133-138). Accordingly, the human proteins of the present invention are contemplated for use in the prevention of preterm delivery.

Aprotinin has been shown to stimulate differentiation of mouse myoblasts in culture (Wells and Strickland, Development, (1994), 120: 3639-3647), a process that is inhibited by TGF β . TGF β exists as an inactive pro-polypeptide which is activated by limited proteolysis. The mechanism of aprotinin action has been proposed to involve inhibition of proteases which process pro-TGF β to the mature active form. TGF β has been shown to be up-regulated in various fibrotic lesions and has long been thought to be a potential target for anti-fibrotic therapies. In a rat model of pulmonary fibrosis for example, TGF- β concentrations paralleled the extent of bleomycin-induced inflammation. Furthermore, plasmin levels in the alveolar macrophage coincided with mature TGF- β levels, and the addition of the plasmin inhibitor

a-2-antiplasmin abrogated the post translational activation of pro-TGF β by the macrophage (Khal et al., (1996), Am. J. Respir. Cell Mol. Biol. 15: 252-259.) The data suggest that plasmin contributes to the formation of active TGF β by alveolar macrophage, and that this process plays a pathologic role in the bleomycin-induced lung inflammation.

In light of these observations, placental bikunin and fragments thereof are contemplated as therapeutics for various fibrotic disorders, including pulmonary, hepatic, renal and dermal (scleroderma) fibrosis.

Aerosilized aprotinin was shown to protect >50% of mice infected with lethal doses of either influenza virus or paramyxovirus (Ovcharenko and Zhirnov, Antiviral Research, (1994), 23: 107-118). A suppression of the development of fatal hemorrhagic bronchopneumonia and a normalization of body weight gain were also noted with aerosilized aprotinin treatment. In light of these observations, placental bikunin and fragments thereof are contemplated as therapeutics for various respiratory related influenza-like diseases.

The human placental bikunin, isolated domains, and other variants of the invention are contemplated for use in the medical/therapeutic applications suggested for native aprotinin or aprotinin analogues with other inhibitory profiles, in particular those which necessitate usage of large doses. These would include diseases for which use of the human protein is indicated by virtue of its ability to inhibit human serine proteases such as trypsin, plasmin, kallikrein, elastase, cathepsin G and proteinase-3, which include and are not limited to: acute pancreatitis (pancreatic elastase and trypsin), inflammation, thrombocytopenia, preservation of platelet function, organ preservation, wound healing, various forms of shock, including shock lung, endotoxin shock and post operative complications; disturbances of blood coagulation such as hyperfibrinolytic hemorrhage; acute and chronic inflammatory reactions, in particular for the therapy and prophylaxis of organ lesions, such as for example pancreatitis and radiation induced enteritis, complex-mediated inflammatory reactions such as immunovasculitis, glomerulonephritis and types of arthritis; collagenoses in particular rheumatoid arthritis; types of arthritis caused by metabolism-related deposits (for example gout); degeneration of the elastic constituents of the connective tissue parts of organs, such as in atherosclerosis (serum elastase) or pulmonary emphysema (neutrophil elastase); adult respiratory distress syndrome, inflammatory bowel disease, and psoriasis.

A major unexpected finding was that the synthetic peptides encoding

bikunin (7-64), and bikunin (102-159), could properly fold into the correct three-dimensional conformation having active protease inhibitor bioactivity (Examples 2 and 1, respectively). Upon folding, each of these fragments of Bikunin underwent a reduction in mass of 6 mass units, consistent with the formation in each case, of three intrachain disulfide bonds between six cysteine residues of each fragment. Another surprising finding is that the synthetic peptides encoding bikunin (7-64), bikunin (102-159), and bikunin (1-170) are highly inhibitory of plasmin and both tissue and plasma kallikrein (Example 4, 3, and 10 respectively). Inhibition of plasmin and kallikrein by Trasylo[®] is thought to be involved in the mechanism by which Trasylo[®] reduces blood loss during open heart surgery. Our unexpected findings of the specificity of the Kunitz domains of the present invention make them suitable therapeutic agents for blood sparing during surgery or trauma where there is significant blood loss, or for any other condition where inhibition of plasmin and/or kallikrein would be beneficial.

Furthermore, we showed in this disclosure (Example 10) that placental bikunin (1-170) is a potent inhibitor of factor XIa and a moderate inhibitor of factor Xa. Factor XIa plays an essential role in the intrinsic pathway of coagulation, serving to interconvert inactive factor IX into active factor IXa. Thus, Placental Bikunin inhibits two key enzymes of the intrinsic pathway, kallikrein and factor XIa. Consistent with these observations, we also showed that placental bikunin (1-170) is a potent inhibitor of the activated partial thromboplastin time, which is a measure of the speed of coagulation driven by the intrinsic pathway. On the other hand, we showed that Placental bikunin (1-170) is an extremely weak inhibitor of the tissue factor VIIa complex, suggesting that it is not important in the regulation of the extrinsic coagulation cascade. Based on these unexpected findings, placental bikunin is contemplated as a medicament for diseases in which activation of the intrinsic pathway of coagulation contributes significantly to the disease mechanism. Examples of such diseases would include post-traumatic shock and disseminated intravascular coagulation.

A significant advantage of the Kunitz domains of the present invention is that they are human proteins, and also less positively charged than Trasylo[®] (Example 1), thereby reducing the risk of kidney damage on administration of large doses of the proteins. Being of human origin, the protein of the instant invention can thus be administered to human patients with significantly reduced risk of undesired immunological reactions as compared to

administration of similar doses of Trasylol®. Furthermore, it was found that bikunin (102-159), bikunin (7-64), and bikunin (1-170) are significantly more potent inhibitors of plasma kallikrein than Trasylol® *in vitro* (Example 3, 4 and 10). Thus bikunin and fragments thereof are expected to be more effective *in vivo* at lowering blood loss in patients.

The amount of serine protease inhibitor administered should be sufficient to provide a supra normal plasma level. For the prophylactic reduction of bleeding during and following coronary aortic by-pass surgery (CABG), the proteins of the instant invention may be used in place of Trasylol® while taking into account the differences in potency. The use of Trasylol® is outlined in the Physicians Desk Reference, (1995), listing for Trasylol® supplement A. Briefly, with the patient in a supine position, the loading dose of placental bikunin, isolated domain or other variant is given slowly over about 20 to 30 minutes, after induction of anesthesia but prior to sternotomy. In general, a total dose of between about 2×10^6 KIU (kallikrein inhibitory units) and 8×10^6 KIU will be used, depending on such factors as patient weight and the length of the surgery. Preferred loading doses are those that contain a total of 1 to 2 million kallikrein inhibitory units (KIU). When the loading dose is complete, it is followed by the constant infusion dose, which is continued until surgery is complete and the patient leaves the operating room. Preferred constant infusion doses are in the range of about 250,000 to 500,000 KIU per hour. The pump prime dose is added to the priming fluid of the cardiopulmonary bypass circuit, by replacement of an aliquot of the priming fluid prior to the institution of the cardiopulmonary bypass. Preferred pump prime doses are those that contain a total of about one to two million KIU.

The proteins of the instant invention are employed in pharmaceutical compositions formulated in the manner known to the art. Such compositions contain active ingredient(s) plus one or more pharmaceutically acceptable carriers, diluents, fillers, binders, and other excipients, depending on the administration mode and dosage form contemplated. Examples of therapeutically inert inorganic or organic carriers known to those skilled in the art include, but are not limited to, lactose, corn starch or derivatives thereof, talc, vegetable oils, waxes, fats, polyols such as polyethylene glycol, water, saccharose, alcohols, glycerin and the like. Various preservatives, emulsifiers, dispersants, flavorants, wetting agents, antioxidants, sweeteners, colorants, stabilizers, salts, buffers and the like can also be added, as required to assist in the stabilization of the formulation or to assist in increasing bioavailability of

the active ingredient(s) or to yield a formulation of acceptable flavor or odor in the case of oral dosing. The inhibitor employed in such compositions may be in the form of the original compound itself, or optionally, in the form of a pharmaceutically acceptable salt. The proteins of the instant invention can be administered alone, or in various combinations, and in combination with other therapeutic compositions. The compositions so formulated are selected as needed for administration of the inhibitor by any suitable mode known to those skilled in the art.

Parenteral administration modes include intravenous (*i.v.*), subcutaneous (*s.c.*), intraperitoneal (*i.p.*), and intramuscular (*i.m.*) routes. Intravenous administration can be used to obtain acute regulation of peak plasma concentrations of the drug as might be needed. Alternatively, the drug can be administered at a desired rate continuously by *i.v.* catheter. Suitable vehicles include sterile, non-pyrogenic aqueous diluents, such as sterile water for injection, sterile-buffered solutions or sterile saline. The resulting composition is administered to the patient prior to and/or during surgery by intravenous injection or infusion.

Improved half-life and targeting of the drug to phagosomes such as neutrophils and macrophage involved in inflammation may be aided by entrapment of the drug in liposomes. It should be possible to improve the selectivity of liposomal targeting by incorporating into the outside of the liposomes ligands that bind to macromolecules specific to target organs/tissues such as the GI tract and lungs. Alternatively, *i.m.* or *s.c.* deposit injection with or without encapsulation of the drug into degradable microspheres (e.g., comprising poly-DL-lactide-co-glycolide) or protective formulations containing collagen can be used to obtain prolonged sustained drug release. For improved convenience of the dosage form it is possible to use an *i.p.* implanted reservoir and septum such as the percuseal system. Improved convenience and patient compliance may also be achieved by use of either injector pens (e.g., the Novo Pin or Q-pen) or needle-free jet injectors (e.g., from Bioject, Mediject or Becton Dickinson). Precisely controlled release can also be achieved using implantable pumps with delivery to the desired site via a cannula. Examples include the subcutaneously implanted osmotic pumps available from ALZA such as the ALZET osmotic pump.

Nasal delivery may be achieved by incorporating the drug into bioadhesive particulate carriers (<200 mm) such as those comprising cellulose, polyacrylate or polycarbophil, in conjunction with suitable absorption

enhancers such as phospholipids or acylcarnitines. Commercially available systems include those developed by Dan Biosys and Scios Nova.

5 Pulmonary delivery represents a nonparenteral mode of administration of the drug to the circulation. The lower airway epithelia are highly permeable to a wide range of proteins of molecular sizes up to about 20 kDa. Micron-sized dry powders containing the medicament in a suitable carrier such as mannitol, sucrose or lactose may be delivered to the distal alveolar surface using dry powder inhalers such as those of Inhale™, Dura™, Fisons (Spinhaler™), and Glaxo (Rotahaler™), or Astra (Turbohaler™) propellant based metered dose inhalers. 10 Solution formulations with or without liposomes may be delivered using ultrasonic nebulizers.

Oral delivery may be achieved by incorporating the drug into tablets, coated tablets, dragées, hard and soft gelatin capsules, solutions, emulsions, suspensions or enteric coated capsules designed to release the drug into the colon where digestive protease activity is low. Examples of the latter include 15 the OROS-CT/Osmet™ system of ALZA, and the PULSINCAP™ system of Scherer Drug Delivery Systems. Other systems use azo-crosslinked polymers that are degraded by colon-specific bacterial azoreductases, or pH sensitive polyacrylate polymers that are activated by the rise in pH in the colon. The above systems may be used in conjunction with a wide range of available absorption enhancers. Rectal delivery may be achieved by incorporating the drug into suppositories. 20

In its preferred medicinal application, for reduction of perioperative blood loss, the preferred mode of administration of the placental bikunin variants of the present invention is parenterally, preferably by *i.v.* route through a central line. 25

The amount of the pharmaceutical composition to be employed will depend on the recipient and the condition being treated. The requisite amount may be determined without undue experimentation by protocols known to those skilled in the art. Alternatively, the requisite amount may be calculated, 30 based on a determination of the amount of target protease such as plasmin or kallikrein which must be inhibited in order to treat the condition. As the active materials contemplated in this invention are deemed to be nontoxic, treatment preferably involves administration of an excess of the optimally required amount of active agent. 35

Additionally, placental bikunin, isolated domains or other variants may be used to isolate natural substances such as its cognate proteases from human

material using affinity based separation methods, as well as to elicit antibodies to the protease that can be further used to explore the tissue distribution and useful functions of Placental bikunin.

5 Searching Human Sequence Data

The existence of a distinct human protein homologous in function to aprotinin, was deduced following a unique analysis of sequence entries to the expressed-sequence-tag data-base (hereafter termed dbEST) at the NCBI (National Center for Biological Information, Maryland). Using the TBlastN
10 algorithm (BLAST, or Basic Local Alignment Search Tool uses the method of Altschul et a., (1990) J. Mol Biol 215: 403-410, to search for similarities between a query sequence and all the sequences in a data-base, protein or nucleic acid in any combination), the data-base was examined for nucleotide sequences bearing homology to the sequence of bovine pre-pro-aprotinin, Trasylol®. This
15 search of numerous clones was selectively narrowed to two particular clones which could possibly encode for a deduced amino acid sequence that would correspond to a human protein homologous in function to aprotinin. The selected nucleic acid sequences were R35464 (SEQ ID NO: 12) and R74593 (SEQ ID NO: 14) that were generated from a human placental nucleic acid library.
20 The translated protein sequence in the longest open reading frame for R35464 (SEQ ID NO: 13) was missing one of the 6 cysteines that are critical for formation of the Kunitz-domain covalent structure, meaning that the nucleic acid sequence of R35464 could not yield a functional inhibitor. Similarly, the longest translated open reading frame from clone R74593 (SEQ ID NO: 15)
25 contained a stop codon 5' to the region encoding the Kunitz like sequence, meaning that this sequence, could not be translated to yield a functional secreted Kunitz domain. The significance of these sequences alone was unclear. It was possible that they represented a) the products of pseudogenes, b) regions of untranslated mRNA, or c) the products of viable mRNA which had been
30 sequenced incorrectly.

Discovery of Human Bikunin

To specifically isolate and determine the actual human sequence, cDNA primers were designed to be capable of hybridizing to sequences located 5' and
35 3' to the segment of cDNA encoding our proposed Kunitz like sequences found within R35464 and R74593. The primers used to amplify a fragment encoding the Kunitz like sequence of R74593 were:

CGAAGCTTCATCTCCGAAGCTCCAGACG (the 3' primer with a HindIII site; SEQ ID NO:33) and AGGATCTAGACAATAATTACCTGACCAAGGA (the 5' primer with an XbaI site; SEQ ID NO:34).

5 These primers were used to amplify by PCR (30 cycles) a 500 base pair product from a human placental cDNA library from Clontech (MATCHMAKER, Cat #HL4003AB, Clontech Laboratories, Palo Alto, CA), which was subcloned into Bluescript-SK+ and sequenced with the T3 primer with a Sequenase™ kit version 2.0. Surprisingly, the sequence of the fragment
10 obtained using our primers was different from the sequence listed in the dbEST data base for clone R74593. In particular, our new sequence contained an additional guanosine base inserted 3' to the putative stop codon, but 5' to the segment encoding the Kunitz-like sequence (Figure 3). The insertion of an additional G shifted the stop codon out of the reading frame for the Kunitz-like domain (G at base pair 114 of the corrected sequence for R74593; Figure 3).

15 Subsequent query of the dbEST for sequences homologous to the Kunitz-like peptide sequence of R74593 yielded H94519 derived from human retina library and N39798. These sequences contained a Kunitz-like sequence that was almost identical to the Kunitz-like domain encoded in R35464 except that it contained all six of the characteristic cysteines. Overlay of each of the
20 nucleotide sequences with that of R74593 (corrected by the insertion of G at b.p. 114) and R35464 was used to obtain a consensus nucleotide sequence for a partial human placental bikunin (SEQ ID NO: 9; Figure 3). The translated consensus sequence yielded an open reading frame extending from residue -18 to +179 (Figure 3; full translation SEQ ID NO: 10) that contained two complete
25 Kunitz-like domain sequences, within the region of amino acid residues 17-64 and 102-159 respectively.

Further efforts attempted to obtain additional 5' sequence by querying dbEST with the sequence of R35464. Possible matches from such searches, that possessed additional 5' sequence were then in turn used to re-query the dbEST.
30 In such an iterative fashion, a series of overlapping 5' sequences were identified which included clones H16866, T66058, R34808, R87894, N40851 and N39876 (Figure 4). Alignment of some of these sequences suggested the presence of a 5' ATG which might serve as a start site for synthesis of the consensus translated protein sequence. From this selected information, it was now possible to
35 selectively screen for, and determine the nucleic acid and polypeptide sequences of a human protein with homologous function to aprotinin.

Re-interrogation of the dbEST revealed a number of new EST entries

shown schematically in Figure 4B. Overlap with these additional ESTs allowed us to construct a much longer consensus oligonucleotide sequence (Figure 4C) that extended both 5' and 3' beyond the original oligonucleotide sequence depicted in Figure 3. In fact, the new sequence of total length 1.6 kilobases
5 extended all the way to the 3' poly-A tail. The increased number of overlapping ESTs at each base-pair position along the sequence improved the level of confidence in certain regions such as the sequence overlapping with the 3' end of EST R74593 (Figure 3). Several overlapping ESTs in this region corroborated two critical base deletions relative to R74593 (located as bold underlined in
10 Figure 4C, map positions 994 and 1005). Translation of the new consensus sequence (Figure 4D) in the bikunin encoding frame yielded a form of placental bikunin that was larger (248 amino acids) than the mature sequence (179 amino acids) encoded from the original consensus (SEQ ID NO: 1), and was terminated by an in-frame stop codon within the oligonucleotide consensus.
15 The size increase was due to a frame shift in the 3' coding region resulting from removal of the two base insertions unique to EST R74593. The frame shift moved the stop codon of the original consensus (Figure 3) out of frame enabling read through into a new frame encoding the additional amino acid sequence. The new translation product (Figure 4D) was identical to the original
20 protein consensus sequence (SEQ ID NO: 1) between residues +1 to +175 (encoding the Kunitz domains), but contained a new C-terminal extension exhibiting a putative 24 residue long transmembrane domain (underlined in Figure 4D) followed by a short 31 residue cytoplasmic domain. The precise sequence around the initiator methionine and signal peptide was somewhat
25 tentative due to considerable heterogeneity amongst the overlapping ESTs in this region.

Analysis of the protein sequence by Geneworks™, highlighted asparagine residues at positions 30 and 67 as consensus sites for putative N-linked glycosylation. Asparagine 30 was not observed during N-terminal
30 sequencing of the full length protein isolated from human placenta, consistent with it being glycosylated.

Cloning of Human Bikunin

The existence of a human mRNA corresponding to the putative human
35 bikunin nucleotide sequence inferred from the analysis of Figure 3, was confirmed as follows. The nucleic acid primer hybridizing 5' to the Kunitz-encoding cDNA sequence of R35464 (b.p. 3-27 of consensus nucleotide

sequence in Figure 3):

GGTCTAGAGGCCGGGTCGTTTCTCGCCTGGCTGGGA

(a 5' primer derived from R35464 sequence with an XbaI site; SEQ ID NO: 35), and the nucleic acid primer hybridizing 3' to the Kunitz encoding sequence of R74593 (b.p. 680-700 of consensus nucleotide sequence in Figure 3), was used to PCR amplify, from a Clontech human placental library, a fragment of the size (ca. 670 b.p) expected from a cDNA consensus nucleotide sequence encoding the placental bikunin sequence of Figure 3 (Shown schematically in Figure 4A).

Using a 5' primer hybridizing to a sequence in R87894 that is 126 b.p 5' to the putative ATG start site discussed above, (shown schematically in Figure 4A at b.p. 110) plus the same 3' primer to R74593 as used above, it was possible to amplify a fragment from a Clontech human placental library of the expected size (approximately 872 b.p) predicted by EST overlay (Shown schematically in Figure 4).

Sequencing of the 872 b.p. fragment showed it to contain nucleotide segment corresponding to b.p. 110 to 218 of EST R87894 at its 5' end and b.p. 310 to 542 of the consensus sequence for placental bikunin inferred from the EST overlay analysis (of Figure 3), at its 3' end. This 3' nucleotide sequence contained all of the Kunitz-like domain encoded by placental bikunin (102-159).

To obtain a cDNA encoding the entire extracellular region of the protein, the following 5' PCR primer:

CACCTGATCGCGAGACCCC (SEQ ID NO: 36)

designed to hybridize to a sequence within EST R34808 was used with the same 3' primer to EST 74593 to amplify (30 cycles) an approximately 780 base-pair cDNA product from the human placental cDNA library. This product was gel purified, and cloned into the TA vector (Invitrogen) for DNA sequencing by the dideoxy method (Sanger F., et al., (1977) Proc. Natl. Acad. Sci (USA), 74: 5463-5467) with the following primers:

Vector Specific: GATTTAGGTGACACTATAG (SP6) (SEQ ID NO: 37)
TAATACGACTCACTATAGGG (T7) (SEQ ID NO: 38)

Gene Specific: TTACCTGACCAAGGAGGAGTGC (SEQ ID NO: 39)
AATCCGCTGCATTCCTGCTGGTG (SEQ ID NO: 40)
CAGTCACTGGGCCTTGCCGT (SEQ ID NO: 41)

The resulting cDNA sequence is depicted in Figure 4E together with its

translation product. At the nucleotide level, the sequence exhibited only minor differences from the consensus EST sequence (Figure 4D). Translation of the sequence yielded a coding sequence containing an in-frame initiator ATG site, signal peptide and mature placental bikunin sequence and transmembrane domain. The translated sequence of the PCR product was missing the last 12 amino acid residues from the cytoplasmic domain as a consequence of the choice of selection of the 3' primer for PCR amplification. This choice of 3' PCR primer (designed based on the sequence of R74593) was also responsible for the introduction of an artifactual S to F mutation at amino acid position 211 of the translated PCR-derived sequence. The signal peptide deduced from translation of the PCR fragment was somewhat different to that of the EST consensus.

To obtain a full length placental bikunin cDNA, the PCR derived product (Figure 4E) was gel purified and used to isolate a non-PCR based full length clone representing the bikunin sequence. The PCR derived cDNA sequence was labeled with ^{32}P -CTP by High Prime (Boehringer Mannheim) and used to probe a placental cDNA Library (Stratagene, UnizapTM λ library) using colony hybridization techniques. Approximately 2×10^6 phage plaques underwent 3 rounds of screening and plaque purification. Two clones were deemed full length (~1.5 kilobases) as determined by restriction enzyme analysis and based on comparison with the size of the EST consensus sequence (see above). Sequencing of one of these clone by the dideoxy method yielded the oligonucleotide sequence depicted in Figure 4F. The translation product from this sequence yielded a protein with inframe initiator methionine, signal peptide and mature placental bikunin sequence. The mature placental bikunin sequence was identical to the sequence of the mature protein derived by translation of the EST consensus although the signal peptide sequence lengths and sequences differed. Unlike the PCR derived product, the cDNA derived by colony hybridization contained the entire ectodomain, transmembrane domain, cytoplasmic domain and in-frame stop codon. In fact, the clone extended all the way to the poly-A tail. The initiator methionine was followed by a hydrophobic signal peptide which was identical to the signal peptide encoded in the PCR derived clone. Subsequently we expressed and purified a soluble fragment of placental bikunin, bikunin (1-170), from Sf9 cells (Example 9), and found it to be a functional protease inhibitor (Example 10). Furthermore, we isolated from human placenta a soluble fragment of placental bikunin which was also an active protease inhibitor (Example 7). Both the natural protein and the form of the protein expressed in Sf9 cells are probably glycosylated at the asparagine

residue at position 30 based on the recoveries of PTH-amino acids during N-terminal sequencing (Examples 7 and 9).

Based on the above observations, it seems that full length placental bikunin has the capacity to exist as a transmembrane protein on the surface of cells as well as a soluble protein. Other transmembrane proteins that contain Kunitz domains are known to undergo proteolytic processing to yield mixtures of soluble and membrane associated forms. These include two forms of the Amyloid Precursor Protein termed APP751 (Esch F., et al., (1990) Science, 248: 1122-1124) and APP 770 (Wang R., et al., (1991), J. Biol Chem, 266: 16960-16964).

Contact activation is a process which is activated by exposure of damaged vascular surfaces to components of the coagulation cascade. Angiogenesis is a process that involves local activation of plasmin at endothelial surfaces. The specificity of placental bikunin and its putative capacity to anchor to cell surfaces, suggest that the physiologic functions of transmembranous placental bikunin may include regulation of contact activation and angiogenesis.

The amino acid sequences for placental bikunin (7-64), bikunin (102-159), and full length placental bikunin (Figure 4F) were searched against the PIR (Vers. 46.0) and PatchX (Vers. 46.0) protein databases as well as the GeneSeq (Vers. 20.0) protein database of patented sequences using the Genetics Computer Group program FastA. Using the Genetics Computer Group program TFASTA (Pearson and Lipman, 1988, Proc. Natl. Acad. Sci. USA 85: 2444-2448), these same protein sequences were searched versus the six-frame translations of the GenBank (Vers. 92.0 with updates to 1/26/96) and EMBL (modified Vers. 45.0) nucleotide databases as well as the GeneSeq (Vers. 20.0) nucleotide database of patented sequences. The EST and STS subsets of GenBank and EMBL were not included in this set of searches. The best matches resulting from these searches contained sequences which were only about 50% identical over their full length to the 58-amino acid protein sequence derived from our analysis of clones R74593 and R35464.

Isolation of Human Bikunin

As mentioned above, synthetic peptides corresponding to bikunin (7-64) and bikunin (102-159) as determined from the translated consensus sequence for bikunin (Figure 3), could be refolded (Examples 2 and 1, respectively) to yield active kallikrein inhibitor protein (Example 4 and 3, respectively). We exploited this unexpected property to devise a purification scheme to isolate

native placental bikunin from human tissue.

Using a purification scheme which employed kallikrein-sepharose affinity chromatography as a first step, highly purified native potent kallikrein inhibitor was isolated. The isolated native human bikunin had an identical N-terminus (sequenced for 50 amino acid residues) as the sequence predicted by the translation of the consensus nucleic acid sequence (Figure 3) amino acid residues +1 to +50 (Example 7). This confirmed for the first time the existence of a novel native kallikrein inhibitor isolated from human placenta.

Known Kunitz-like domains are listed below. Residues believed to be making contact with target proteases are highlighted as of special interest (bold/underlined). These particular residues are named positions Xaa¹⁻¹⁶ for specific reference as shown by label Xaa below:

| 15 | Xaa | | | | | | | | | | 1 1 111 | | 1 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|----|-----|-----|---|---|---|---|---|---|---|----|---------|---|-----|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 0 | 1 | 234 | 5 | 6 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | 1) | I | H | D | F | C | L | V | S | K | V | G | R | C | R | A | S | M | P | R | W | Y | N | V | T | D | G | S | C | Q | L | F | V | Y | G | G | C | G | G | N | S | N | N | Y | L | T | K | E | E | C | L | K | K | C | A | T | V | | |
| | 2) | Y | E | E | Y | C | T | A | N | A | V | T | G | P | C | R | A | S | F | P | R | W | Y | F | D | V | E | R | N | S | C | N | F | N | F | Y | G | G | C | R | G | N | K | N | S | Y | R | S | E | E | A | C | M | L | R | C | F | R | Q |
| | 3) | - | H | S | F | C | A | F | K | A | D | D | G | P | C | K | A | I | M | K | R | F | F | N | I | F | T | R | Q | C | E | F | F | Y | G | G | C | E | G | N | Q | N | R | F | E | S | L | E | E | C | K | K | M | C | T | R | D | | |
| | 4) | - | P | D | F | C | F | L | E | E | D | P | G | I | C | R | G | Y | I | T | R | Y | F | Y | N | N | Q | T | K | Q | C | E | R | F | K | Y | G | G | C | L | G | N | M | N | F | E | T | L | E | E | C | K | N | I | C | E | D | G | |
| 20 | 5) | - | P | S | W | C | L | T | P | A | D | R | G | L | C | R | A | N | E | N | R | F | Y | N | S | V | I | G | K | C | R | P | F | K | Y | S | G | C | G | G | N | E | N | N | F | T | S | K | Q | E | C | L | R | A | C | K | K | G | |
| | 6) | - | A | E | I | C | L | L | P | L | D | Y | G | P | C | R | A | L | L | L | R | Y | Y | R | Y | R | T | Q | S | C | R | Q | F | L | Y | G | G | C | E | G | N | A | N | N | F | Y | T | W | E | A | C | D | D | A | C | W | R | I | |
| | 7) | - | P | S | F | C | Y | S | P | K | D | E | G | L | C | S | A | N | V | T | R | Y | Y | F | N | P | R | Y | R | T | C | D | A | F | T | Y | T | G | C | G | G | N | D | N | N | F | V | S | R | E | D | C | K | R | A | C | A | K | A |
| | 8) | - | K | A | V | C | S | Q | E | A | M | T | G | P | C | R | A | V | M | P | R | T | T | F | D | L | S | K | G | K | C | V | R | F | I | T | G | C | G | G | N | R | N | N | F | E | S | E | D | Y | C | M | A | V | C | K | A | M | |
| | 9) | R | P | D | F | C | L | E | P | P | Y | T | G | P | C | R | A | I | I | R | Y | F | Y | N | A | K | A | G | L | C | Q | L | T | F | V | Y | G | C | R | A | K | R | N | N | F | K | S | A | E | D | C | M | R | T | C | G | G | A | |
| 25 | 10) | --- | C | Q | L | G | Y | S | A | G | P | C | M | G | M | T | S | R | Y | F | Y | N | G | T | S | M | A | C | E | T | F | T | F | Q | Y | G | C | M | G | N | G | N | F | V | T | E | K | E | C | L | Q | T | C | | | | | | |
| | 11) | V | A | A | C | N | L | P | I | V | R | G | P | C | R | A | F | I | Q | L | W | A | F | D | A | V | K | G | K | C | V | L | F | P | Y | G | G | C | Q | G | N | G | N | K | F | Y | S | E | K | E | C | R | E | Y | C | G | V | P | |
| | 12) | - | E | V | C | C | S | E | Q | A | E | T | G | P | C | R | A | M | I | S | R | W | Y | F | D | V | T | E | G | K | C | A | P | F | F | Y | G | G | C | G | G | N | R | N | N | F | D | T | E | E | Y | C | M | A | V | C | G | S | A |
| | 13) | --- | C | K | L | P | K | D | E | G | T | C | R | D | F | I | L | K | W | Y | Y | D | P | N | T | K | S | C | A | R | F | W | Y | G | G | C | G | G | N | E | N | K | F | G | S | Q | K | E | C | E | K | V | C | | | | | | |
| | 14) | - | P | N | V | C | A | F | P | M | E | K | G | P | C | Q | T | Y | M | T | R | W | F | F | N | F | E | T | G | E | C | E | L | F | A | Y | G | C | G | G | N | S | N | N | F | L | R | K | E | K | C | E | K | F | C | K | E | T | |

Where sequence number 1) is Bikunin (7-64) (SEQ ID NO: 4); sequence 2) is Bikunin (102-159) (SEQ ID NO: 6); sequence 3) is Tissue factor pathway inhibitor precursor 1 (SEQ ID NO: 18); sequence 4) is Tissue factor pathway inhibitor precursor 1 (SEQ ID NO: 19); sequence 5) is Tissue factor pathway inhibitor precursor (SEQ ID NO: 20); sequence 6) is Tissue factor pathway inhibitor precursor 2 (SEQ ID NO: 21); sequence 7) is Tissue factor pathway inhibitor precursor 2 (SEQ ID NO: 22); sequence 8) is Amyloid precursor protein homologue (SEQ ID NO: 23); sequence 9) is Aprotinin (SEQ ID NO: 24);

sequence 10) is Inter- α -trypsin inhibitor precursor (SEQ ID NOs: 25); sequence 11) is Inter- α -trypsin inhibitor precursor (SEQ ID NOs: 26); sequence 12) is Amyloid precursor protein (SEQ ID NO: 27); sequence 13) is Collagen α -3(VI) precursor (SEQ ID NO: 28); and sequence 14) is HKI-B9 (SEQ ID NO: 29).

5 It can be seen that Placental Bikunin (7-64) and (102-159) each have the same number (six) and spacing of cysteine residues as is found in members of the Kunitz class of serine protease inhibitors. The precise bonding of cysteine residues to form the three intrachain disulfide bonds is known and invariant for all previously known Kunitz family members (Laskowski, M et al., 1980, Ann.
10 Rev. Biochem. 49:593-626). Based on this known bonding pattern and the fact that the folding of Placental Bikunin (7-64) and (102-159) into active protease inhibitors is accompanied by a mass reduction consistent with the formation of three intrachain disulfide bonds (Examples 2 and 1), it is highly probable that the disulfide bonding within the Kunitz domains of Placental Bikunin occur
15 between cysteine residues: C11 and C61; C20 and C44; C36 and C57; C106 and C156; C115 and C139; C131 and C152. Furthermore, this pattern of disulfide bonding is highly probable in larger forms of Placental Bikunin containing both Kunitz domains since such forms of the protein are also active serine protease inhibitors and because N-terminal sequencing (Example 7) of native Placental
20 Bikunin for 50 cycles yielded a sequence that was silent at positions where the cysteine residues were expected.

The placental bikunin, isolated domains or other variants of the present invention may be produced by standard solid phase peptide synthesis using either t-Boc chemistry as described by Merrifield R.B. and Barany G., in: The
25 peptides, Analysis, Synthesis, Biology, 2, Gross E. et al., Eds. Academic Press (1980) Chapter 1; or using F-moc chemistry as described by Carpino L.A., and Han G.Y., (1970) J. Amer Chem Soc., 92, 5748-5749, and illustrated in Example 2. Alternatively, expression of a DNA encoding the placental bikunin variant may be used to produce recombinant placental bikunin variants.

30 The invention also relates to DNA constructs that encode the Placental bikunin protein variants of the present invention. These constructs may be prepared by synthetic methods such as those described in Beaucage S.L. and Caruthers M.H., (1981) Tetrahedron Lett, 22, pp1859-1862; Matteucci M.D and Caruthers M.H., (1981), J. Am. Chem. Soc. 103, p 3185; or from genomic or
35 cDNA which may have been obtained by screening genomic or cDNA libraries with cDNA probes designed to hybridize with placental bikunin encoding DNA sequence. Genomic or cDNA sequence can be modified at one or more

sites to obtain cDNA encoding any of the amino acid substitutions or deletions described in this disclosure.

The instant invention also relates to expression vectors containing the DNA constructs encoding the placental bikunin, isolated domains or other
5 variants of the present invention that can be used for the production of recombinant placental bikunin variants. The cDNA should be connected to a suitable promoter sequence which shows transcriptional activity in the host cell of choice, possess a suitable terminator and a poly-adenylation signal. The cDNA encoding the placental bikunin variant can be fused to a 5' signal peptide
10 that will result in the protein encoded by the cDNA to undergo secretion. The signal peptide can be one that is recognized by the host organism. In the case of a mammalian host cell, the signal peptide can also be the natural signal peptide present in full length placental bikunin. The procedures used to prepare such vectors for expression of placental bikunin variants are well known in the art
15 and are for example described in Sambrook et al., *Molecular Cloning: A laboratory Manual*, Cold Spring Harbor, New York, (1989).

The instant invention also relates to transformed cells containing the DNA constructs encoding the placental bikunin, isolated domains or other
20 variants of the present invention that can be used for the production of recombinant placental bikunin variants. A variety of combinations of expression vector and host organism exist which can be used for the production of the placental bikunin variants. Suitable host cells include baculovirus infected Sf9 insect cells, mammalian cells such as BHK, CHO, Hela and C-127, bacteria such as *E. coli*, and yeasts such as *Saccharomyces cerevisiae*. Methods
25 for the use of mammalian, insect and microbial expressions systems needed to achieve expression of placental bikunin are well known in the art and are described, for example, in Ausubel F.M et al., *Current Protocols in Molecular Biology*, John Wiley & Sons (1995), Chapter 16. For fragments of placental bikunin containing a single Kunitz inhibitor domain such as bikunin (7-64) and
30 (102-159), yeast and *E. coli* expression systems are preferable, with yeast systems being most preferred. Typically, yeast expression would be carried out as described in US patent 5,164,482 for aprotinin variants and adapted in Example 5 of the present specification for placental bikunin (102-159). *E. coli* expression could be carried out using the methods described in US patent
35 5,032,573. Use of mammalian and yeast systems are most preferred for the expression of larger placental bikunin variants containing both inhibitor domains such as the variant bikunin (7-159).

DNA encoding variants of placental bikunin that possess amino acid substitution of the natural amino sequence can be prepared for expression of recombinant protein using the methods of Kunkel T.A., (1985) Proc. Natl. Acad. Sci USA 82: 488-492. Briefly, the DNA to be mutagenized is cloned into a single
5 stranded bacteriophage vector such as M13. An oligonucleotide spanning the region to be changed and encoding the substitution is hybridized to the single stranded DNA and made double stranded by standard molecular biology techniques. This DNA is then transformed into an appropriate bacterial host and verified by dideoxynucleotide sequencing. The correct DNA is then cloned
10 into the expression plasmid. Alternatively, the target DNA may be mutagenized by standard PCR techniques, sequenced, and inserted into the appropriate expression plasmid.

The following particular examples are offered by way of illustration, and not limitation, of certain aspects and preferred embodiments of the instant
15 invention.

Example 1

Preparation of synthetic placental bikunin (102-159)

Materials and methods/Reagents used. The fluorogenic substrate Tos-Gly-Pro-Lys-AMC was purchased from Bachem BioScience Inc (King of
20 Prussia, PA). PNGB, Pro-Phe-Arg-AMC, Ala-Ala-Pro-Met-AMC, bovine trypsin (type III), human plasma kallikrein, and human plasmin were from Sigma (St. Louis, MO).

Recombinant aprotinin (Trasylol®) was from Bayer AG (Wuppertal, Germany). Pre-loaded Gln Wang resin was from Novabiochem (La Jolla, CA). Thioanisole, ethanedithiol and t-butyl methyl ether was from Aldrich
25 (Milwaukee, WI).

Quantification of functional placental bikunin (7-64) and (102-159)

The amount of trypsin inhibitory activity present in the refolded sample at various stages of purification was measured using GPK-AMC as a substrate. Bovine trypsin (200 pmoles) was incubated for 5 min at 37°C with bikunin (7-64) or (102-159), from various stages of purification, in buffer A (50 mM Hepes, pH 7.5, 0.1 M NaCl, 2 mM CaCl₂ and 0.01% triton X-100). GPK-AMC was
30 added (20 µM final) and the amount of coumarin produced was determined by measuring the fluorescence (ex = 370 nm, em = 432 nm) on a Perkin-Elmer LS-50B fluorimeter over a 2 min. period. For samples being tested the % inhibition

for each was calculated according to equation 1; where R_0 is the rate of fluorescence increase in the presence of inhibitor and R_1 is the rate determined in the absence of added sample. One unit of activity for the inhibitor is defined as the amount needed to achieve 50% inhibition in the assay using the conditions as described.

$$\% \text{ inhibition} = 100 \times [1 - R_0/R_1] \quad (1)$$

Synthesis. Placental bikunin (102-159) was synthesized on an Applied Biosystems model 420A peptide synthesizer using NMP-HBTU Fmoc chemistry. The peptide was synthesized on pre loaded Gln resin with an 8-fold excess of amino acid for each coupling. Cleavage and deprotection was performed in 84.6% trifluoroacetic acid (TFA), 4.4% thioanisole, 2.2% ethanedithiol, 4.4% liquified phenol, and 4.4% H_2O for 2 hours at room temperature. The crude peptide was precipitated, centrifuged and washed twice in t-butyl methyl ether. The peptide was purified on a Dynamax 60A C18 reverse-phase HPLC column using a TFA/acetonitrile gradient. The final preparation (61.0 mg) yielded the correct amino acid composition and molecular mass by Electrospray mass spectroscopy ($MH^+ = 6836.1$; calcd = 6835.5) for the predicted sequence:

YEEYCTANAV TGPCRASFP R WYFDVERN SC NNFIYGGCRG NKNSYRSEEA
CMLRCFRQ (SEQ ID NO: 6)

Purification. Refolding of placental bikunin (102-159) was performed according to the method of Tam et al., (J. Am. Chem. Soc. 1991, 113: 6657-62). A portion of the purified peptide (15.2 mg) was dissolved in 4.0 ml of 0.1 M Tris, pH 6.0, and 8 M urea. Oxidation of the disulfides was accomplished by dropwise addition of a solution containing 23% DMSO, and 0.1 M Tris, pH 6.0 to obtain a final concentration of 0.5 mg/ml peptide in 20% DMSO, 0.1 M Tris, pH 6.0, and 1 M urea. The solution was allowed to stir for 24 hr at 25°C after which it was diluted 1:10 in buffer containing 50 mM Tris, pH 8.0, and 0.1 M NaCl. The material was purified using a kallikrein affinity column made by covalently attaching 30 mg of bovine pancreatic kallikrein (Bayer AG) to 3.5 mls of CNBr activated Sepharose (Pharmacia) according to the manufacturers instructions. The refolded material was loaded onto the affinity column at a flow rate of 1 ml/min and washed with 50 mM Tris, pH 8.0, and 0.1 M NaCl until absorbance at 280 nm of the wash could no longer be detected. The

column was eluted with 3 volumes each of 0.2 M acetic acid, pH 4.0 and 1.7. Active fractions were pooled (see below) and the pH of the solution adjusted to 2.5. The material was directly applied to a Vydac C18 reverse-phase column (5 micron, 0.46 x 25 cm) which had been equilibrated in 22.5% acetonitrile in 0.1% TFA. Separation was achieved using a linear gradient of 22.5 to 40% acetonitrile in 0.1% TFA at 1.0 ml/min over 40 min. Active fractions were pooled, lyophilized, redissolved in 0.1% TFA, and stored at -20°C until needed.

Results. Synthetic placental bikunin (102-159) was refolded using 20% DMSO as the oxidizing agent as described above, and purified by a 2-step purification protocol as shown below, to yield an active trypsin inhibitor (Table 1 below).

Table 1
Purification table for the isolation of synthetic placental bikunin (102-159)

| TABLE 1 | | | | | | |
|---------------------|----------|---------------------|------|------------------------|------------|-------|
| Purification Step | Vol (ml) | mg/ml | mg | Units ^c (U) | SpA (U/mg) | Yield |
| 8.0 M Urea | 4.0 | 3.75 ^a | 15.0 | 0 | 0 | - |
| 20% DMSO | 32.0 | 0.47 ^a | 15.0 | 16,162 | 1,078 | 100 |
| Kallikrein affinity | 9.8 | 0.009 ^b | 0.09 | 15,700 | 170,000 | 97 |
| C18 | 3.0 | 0.013 ^{ab} | 0.04 | 11,964 | 300,000 | 74 |

^aProtein determined by AAA.

^bProtein determined by OD280 nm using the extinction coefficient determined for the purified protein ($1.7 \times 10^4 \text{ Lmol}^{-1} \text{ cm}^{-1}$).

^cOne Unit is defined as the amount of material required to inhibit 50% of trypsin activity in a standard assay.

Chromatography of the crude refolded material over an immobilized bovine pancreatic kallikrein column selectively isolated 6.0% of the protein and 97% of the trypsin inhibitory activity present. Subsequent chromatography using C18 reverse-phase yielded a further purification of 2-fold, with an overall recovery of 74%. On RPHPLC, the reduced and refolded placental bikunin (102-159), exhibited elution times of 26.3 and 20.1 minutes, respectively. Mass spectroscopy analysis of the purified material revealed a molecular mass of 6829.8; a loss of 6 mass units from the starting material. This demonstrates the complete formation of the 3 disulfides predicted from the peptide sequence.

The isoelectric points of the purified, refolded synthetic placental bikunin (102-159) was determined using a Multiphor II Electrophoresis System (Pharmacia) run according to the manufacturers suggestions, together with pI standards, using a precast Ampholine® PAGplate (pH 3.5 to 9.5) and focused for 1.5 hrs. After staining, the migration distance from the cathodic edge of the gel to the different protein bands was measured. The pI of each unknown was determined by using a standard curve generated by a plot of the migration distance of standards versus the corresponding pI's. With this technique, the pI of placental bikunin (102-159) was determined to be 8.3, in agreement with the value predicted from the amino acid sequence. This is lower than the value of 10.5 established for the pI of aprotinin. (Tenstad et al., 1994, Acta Physiol. Scand. 152: 33-50).

Example 2

Preparation of synthetic placental bikunin (7-64)

Placental bikunin (7-64) was synthesized, refolded and purified essentially as described for placental bikunin (102-159) but with the following modifications: during refolding, the synthetic peptide was stirred for 30 hr as a solution in 20% DMSO at 25°C; purification by C18 RP-HPLC was achieved with a linear gradient of 25 to 45% acetonitrile in 0.1% TFA over 40 min (1ml/min). Active fractions from the first C18 run were reapplied to the column and fractionated with a linear gradient (60 min, 1 ml/min) of 20 to 40% acetonitrile in 0.1% TFA.

Results. The final purified reduced peptide exhibited an MH⁺ = 6563, consistent with the sequence:

IHDFCLVSKV VGRCRASMPR WWYNVTDGSC QLFVYGGCDG NSNNYLTKEE
CLKKCATV (SEQ ID NO: 4)

The refolding and purification yielded a functional Kunitz domain that was active as an inhibitor of trypsin (Table 2 below).

Table 2A

Purification table for the isolation of synthetic placental bikunin (7-64)

| TABLE 2A | | | | | | |
|----------------------|----------|-------|------|-----------|------------|-------|
| Purification Step | Vol (ml) | mg/ml | mg | Units (U) | SpA (U/mg) | Yield |
| 8.0 M Urea | 8.0 | 2.5 | 20.0 | 0 | 0 | - |
| 20% DMSO | 64.0 | 0.31 | 20.0 | 68,699 | 3,435 | 100 |
| Kall affinity pH 4.0 | 11.7 | 0.10 | 1.16 | 43,333 | 36,110 | 62 |
| Kall affinity pH 1.7 | 9.0 | 0.64 | 5.8 | 4972 | 857 | 72 |
| C18-1 | 4.6 | 0.14 | 0.06 | 21,905 | 350,143 | 31.9 |
| C18-2 | 1.0 | 0.08 | 0.02 | 7,937 | 466,882 | 11.5 |

5 The purified refolded protein exhibited an $MH^+ = 6558$, i.e. 5 ± 1 mass units less than for the reduced peptide. This demonstrates that refolding caused the formation of at least one appropriate disulfide bond.

10 The pI of placental bikunin (7-64) was determined using the methods employed to determine the pI of placental bikunin (102-159). Placental bikunin (7-64) exhibited a pI that was much higher than the predicted value (pI = 7.9). Refolded placental bikunin (7-64) migrated to the cathodic edge of the gel (pH 9.5) and an accurate pI could not be determined under these conditions.

Continued Preparation of synthetic placental bikunin (7-64)

15 Because the synthetic placental bikunin (7-64) may not have undergone complete deprotection prior to purification and refolding, refolding was repeated using protein which was certain to be completely deprotected. Placental bikunin (7-64) was synthesized, refolded and purified essentially as described for placental bikunin (102-159) but with the following modifications:
 20 during refolding, the synthetic peptide (0.27 mg/ml) was stirred for 30 hr as a solution in 20% DMSO at 25 C; purification by C18 RP-HPLC was achieved with a linear gradient of 22.5 to 50% acetonitrile in 0.1% TFA over 40 min (1 ml/min).

25 **Results.** The final purified reduced peptide exhibited an $MH^+ = 6567.5$, consistent with the sequence:

IHDFCLVSKV VGRCRASMPRW WYNVTDGSC QLFVYGGCDG NSNNYLTKEE
 CLKKCATV (SEQ ID NO: 4)

30 The refolding and purification yielded a functional Kunitz domain that was as active as an inhibitor of trypsin (Table 2B below).

Table 2B

Purification table for the isolation of synthetic placental bikunin (7-64)

| TABLE 2B | | | | | | |
|----------------------------|----------|-------|------|-----------|------------|-------|
| Purification Step | Vol (ml) | mg/ml | mg | Units (U) | SpA (U/mg) | Yield |
| 8.0 M Urea | 4.9 | 2.1 | 10.5 | 0 | 0 | - |
| 20% DMSO | 39.0 | 0.27 | 10.5 | 236,000 | 22,500 | 100 |
| Kallikrein Affinity (pH 2) | 14.5 | 0.3 | 0.43 | 120,000 | 279,070 | 50.9 |
| C18 Reverse-Phase | 0.2 | 1.2 | 0.24 | 70,676 | 294,483 | 30.0 |

5

The purified refolded protein exhibited an $MH^+ = 6561.2$, i.e. 6.3 mass units less than for the reduced peptide. This demonstrates that refolding caused the formation of the expected three disulfide bonds.

10 The pI of refolded placental bikunin (7-64) was determined using the methods employed to determine the pI of placental bikunin (102-159). Refolded placental bikunin (7-64) exhibited a pI of 8.85, slightly higher than the predicted value (pI = 7.9).

Example 315 **In vitro specificity of functional placental bikunin fragment (102-159)**

Proteases. Bovine trypsin, human plasmin, and bovine pancreatic kallikrein quantitation was carried out by active site titration using p-nitrophenyl p'-guanidinobenzoate HCl as previously described (Chase, T., and Shaw, E., (1970) Methods Enzymol., 19: 20-27). Human kallikrein was
20 quantitated by active site titration using bovine aprotinin as a standard and PFR-AMC as a substrate assuming a 1:1 complex formation. The K_m for GPK-AMC with trypsin and plasmin under the conditions used for each enzyme was 29 μM and 726 μM , respectively; the K_m for PFR-AMC with human plasma kallikrein and bovine pancreatic kallikrein was 457 μM and 81.5 μM ,
25 respectively; the K_m for AAPR-AMC with elastase was 1600 μM . Human tissue kallikrein (Bayer, Germany) quantification was carried out by active site titration using p-nitrophenyl p'-guanidinobenzoate HCl as previously described (Chase, T., and Shaw, E., (1970) Methods Enzymol. 19: 20-27).

30 **Inhibition Kinetics:** The inhibition of trypsin by placental bikunin (102-159) or aprotinin was measured by the incubation of 50 pM trypsin with

placental bikunin (102-159) (0-2 nM) or aprotinin (0-3 nM) in buffer A in a total volume of 1.0 ml. After 5 min. at 37°C, 15 µl of 2 mM GPK-AMC was added and the change in fluorescence (as above) was monitored. The inhibition of human plasmin by placental bikunin (102-159) and aprotinin was determined with plasmin (50 pM) and placental bikunin (102-159) (0-10 nM) or aprotinin (0-4 nM) in buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, and 0.02% triton x-100. After 5 min. incubation at 37°C, 25 µl of 20 mM GPK-AMC was added and the change in fluorescence monitored. The inhibition of human plasma kallikrein by placental bikunin (102-159) or aprotinin was determined using kallikrein (2.5 nM) and placental bikunin (102-159) (0-3 nM) or aprotinin (0-45 nM) in 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 0.02% triton x-100. After 5 min. at 37°C 15 µl of 20 mM PFR-AMC was added and the change in fluorescence monitored. The inhibition of bovine pancreatic kallikrein by placental bikunin (102-159) and aprotinin was determined in a similar manner with kallikrein (92 pM), placental bikunin (102-159) (0-1.6 nM) and aprotinin (0-14 pM) and a final substrate concentration of 100 µM. The apparent inhibition constant K_i^* was determined using the nonlinear regression data analysis program Enzfitter software (Biosoft, Cambridge, UK): The kinetic data from each experiment were analyzed in terms of the equation for a tight binding inhibitor:

$$V_i/V_o = 1 - (E_o + I_o + K_i^* - [(E_o + I_o + K_i^*)^2 - 4 E_o I_o]^{1/2}) / 2E_o \quad (2)$$

where V_i/V_o is the fractional enzyme activity (inhibited vs. uninhibited rate), and E_o and I_o are the total concentrations of enzyme and inhibitor, respectively. K_i values were obtained by correcting for the effect of substrate according to the equation:

$$K_i = K_i^* / (1 + [S_o]/K_m) \quad (3)$$

(Boudier, C., and Bieth, J. G., (1989) *Biochim Biophys Acta.*, 995: 36-41)

For the inhibition of human neutrophil elastase by placental bikunin (102-159) and aprotinin, elastase (19 nM) was incubated with placental bikunin (102-159) (150 nM) or aprotinin (0-7.5 µM) in buffer containing 0.1 M Tris-HCl (pH 8.0), and 0.05% triton X-100. After 5 min at 37°C, AAPM-AMC (500 µM or 1000 µM) was added and the fluorescence measured over a two-minute period. K_i values were determined from Dixon plots of the form $1/V$ versus $[I]$

performed at two different substrate concentrations (Dixon et al., 1979).

The inhibition of human tissue kallikrein by aprotinin, placental bikunin fragment (7-64) or placental bikunin fragment (102-159) was measured by the incubation of 0.35 nM human tissue kallikrein with placental bikunin (7-64) (0-40 nM) or placental bikunin (102-159) (0-2.5 nM), or aprotinin (0-0.5 nM) in a 1 ml reaction volume containing 50 mM Tris-HCl buffer pH 9.0, 50 mM NaCl, and 0.1% triton x-100. After 5 min. at 37°C, 5 ul of 2 mM PFR-AMC was added achieving 10 uM final and the change in fluorescence monitored. The K_m for PFR-AMC with human tissue kallikrein under the conditions employed was 5.7 uM. The inhibition of human factor Xa (American Diagnostica, Inc, Greenwich, CT) by synthetic placental bikunin (102-159), recombinant placental bikunin, and aprotinin was measured by the incubation of 0.87 nM human factor Xa with increasing amounts of inhibitor in buffer containing 20 mM Tris (pH 7.5), 0.1 M NaCl, and 0.1% BSA. After 5 min. at 37°C, 30 ul of 20 mM LGR-AMC (Sigma) was added and the change in fluorescence monitored. The inhibition of human urokinase (Sigma) by Kunitz inhibitors was measured by the incubation of urokinase (2.7 ng) with inhibitor in a total volume of 1 ml buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 0.1% Triton x-100. After 5 min. at 37°C, 35 ul of 20 mM GGR-AMC (Sigma) was added and the change in fluorescence monitored. The inhibition of Factor XIa (from Enzyme Research Labs, Southbend, IN) was measured by incubating FXIa (0.1 nM) with either 0 to 800 nM placental bikunin (7-64), 0 to 140 nM placental bikunin (102-159) or 0 to 40 uM aprotinin in buffer containing 50 mM Hepes pH 7.5, 100 mM NaCl, 2 mM $CaCl_2$, 0.01% triton x-100, and 1% BSA in a total volume of 1 ml. After 5 min at 37 C, 10 ul of 40 mM Boc-Glu(OBzl)-Ala-Arg-AMC (Bachem Biosciences, King of Prussia, PA) was added and the change in fluorescence monitored.

Results: A direct comparison of the inhibition profiles of placental bikunin (102-159) and aprotinin was made by measuring their inhibition constants with various proteases under identical conditions. The K_i values are listed in Table 3 below.

Table 3
Ki values for the inhibition of various proteases by bikunin (102-159)

| TABLE 3 | | | | |
|---|---------------------------------|----------------------|------------------------------|------------|
| Protease (concentration) | bikunin (102-159) Ki (nM) | Aprotinin Ki (nM) | Substrate (concentration) | Km (mM) |
| Trypsin (48.5 pM) | 0.4 | 0.8 | GPK-AMC (0.03 mM) | 0.022 |
| Chymotrypsin (5 nM) | 0.24 | 0.86 | AAPF-pNA (0.08 mM) | 0.027 |
| Bovine Pancreatic Kallikrein (92.0 pM) | 0.4 | 0.02 | PFR-AMC (0.1 mM) | 0.08 |
| Human Plasma Kallikrein (2.5 nM) | 0.3 | 19.0 | PFR-AMC (0.3 mM) | 0.46 |
| Human Plasmin (50 pM) | 1.8 | 1.3 | GPK-AMC (0.5 mM) | 0.73 |
| Human Neutrophil Elastase (19 nM) | 323.0 | 8500.0 | AAPM-AMC (1.0 μM) | 1.6 |
| Factor XIIa | >300.0 | 12,000.0 | PFR-AMC (0.2 μM) | 0.35 |
| Human Tissue Kallikrein (0.35 nM) | 0.13 | 0.004 | PFR-AMC (10 μM) | 0.0057 |
| factor Xa (0.87 nM) | 274 | N.I. at 3 μM | LGR-AMC (0.6 mM) | N.D. |
| urokinase | 11000 | 4500 | GGR-AMC (0.7 mM) | N.D. |
| factor XIa (0.1 nM) | 15 | 288 | E(OBz)AR-AMC (0.4 mM) | 0.46 |

5 Placental bikunin (102-159) and aprotinin inhibit bovine trypsin and human plasmin to a comparable extent under the conditions employed. Aprotinin inhibited elastase with a K_i of 8.5 μM. Placental bikunin (102-159) inhibited elastase with a K_i of 323 nM. The K_i value for the placental bikunin (102-159) inhibition of bovine pancreatic kallikrein was 20-fold higher than that of aprotinin inhibition. In contrast, placental bikunin (102-159) is a more potent inhibitor of human plasma kallikrein than aprotinin and binds with a 56-fold higher affinity.

10 Because placental bikunin (102-159) is greater than 50 times more potent than Trasylol® as an inhibitor of kallikrein, smaller amounts of human placental bikunin, or fragments thereof (i.e. placental bikunin (102-159)) are needed than Trasylol® in order to maintain the effective patient doses of inhibitor in KIU. This reduces the cost per dose of the drug and reduces the likelihood of adverse nephrotoxic effects upon re-exposure of the medicament to patients. Furthermore, the protein is human derived, and thus much less immunogenic in man than aprotinin which is derived from cows. This results in significant reductions in the risk of incurring adverse immunologic events upon re-exposure of the medicament to patients.

Example 4**In vitro specificity of functional placental bikunin fragment (7-64)**

In vitro specificity of functional human placental bikunin (7-64) was determined using the materials and methods as described in the Examples above.

Results: The table below shows the efficacy of placental bikunin (7-64) as an inhibitor of various serine proteases *in vitro*. Data is shown compared against data obtained for screening inhibition using either placental bikunin (102-159), or aprotinin (Trasylol®).

Table 4 A
Ki values for the inhibition of various proteases by bikunin(7-64)

| TABLE 4A | | | |
|---|--------------------------|----------------------|------------------------------|
| Protease (concentration) | bikunin(7-64) Ki (nM) | Aprotinin Ki (nM) | bikunin (102-159) Ki (nM) |
| Trypsin (48.5 pM) | 0.17 | 0.8 | 0.4 |
| Bovine Pancreatic Kallikrein (92.0 pM) | 0.4 | 0.02 | 0.4 |
| Human Plasma Kallikrein (2.5 nM) | 2.4 | 19.0 | 0.3 |
| Human Plasmin (50 pM) | 3.1 | 13 | 1.8 |
| Bovine chymotrypsin (5 nM) | 0.6 | 0.9 | 0.2 |
| Factor XIIa | >300 | 12000 | >300 |
| elastase | >100 | 8500 | 323 |

The results show that the amino acid sequence encoding placental bikunin (7-64) can be refolded to obtain an active serine protease inhibitor that is effective against at least four trypsin-like serine proteases.

Table 4B below also shows the efficacy of refolded placental bikunin (7-64) as an inhibitor of various serine proteases *in vitro*. Refolded placental bikunin (7-64) was prepared from protein that was certain to be completely deprotected prior to purification and refolding. Data is shown compared against data obtained for screening inhibition using either placental bikunin (102-159), or aprotinin (Trasylol®).

Table 4B**Ki values for the inhibition of various proteases by refolded bikunin (7-64)**

| TABLE 4B | | | |
|-------------------------------------|---------------------------|----------------------|------------------------------|
| Protease (concentration) | bikunin (7-64) Ki (nM) | Aprotinin Ki (nM) | bikunin (102-159) Ki (nM) |
| Trypsin (50 pM) | 0.2 | 0.8 | 0.3 |
| Human Plasma Kallikrein (0.2 nM) | 0.7 | 19.0 | 0.7 |
| Human Plasmin (50 pM) | 3.7 | 1.3 | 1.8 |
| Factor XIIa | not done | 12,000 | 4,500 |
| Factor XIa (0.1 nM) | 200 | 288 | 15 |
| Human Tissue Kallikrein | 2.3 | 0.004 | 0.13 |

5 Surprisingly, placental bikunin (7-64) was more potent than aprotinin at inhibiting human plasma kallikrein, and at least similar in efficacy as a plasmin inhibitor. These data show that placental bikunin (7-64) is at least as effective as aprotinin, using *in vitro* assays, and that one would expect better or similar potency *in vivo*.

10

Example 5**Expression of placental bikunin variant (102-159) in yeast**

15 The DNA sequence encoding placental bikunin 102-159 (SEQ ID NO: 6) was generated using synthetic oligonucleotides. The final DNA product consisted (5' to 3') of 15 nucleotides from the yeast α -mating factor propeptide sequence fused to the in-frame cDNA sequence encoding placental bikunin (102-159), followed by an in-frame stop codon. Upon cloning into a yeast expression vector pS604, the cDNA would direct the expression of a fusion protein comprising an N-terminal yeast α -mating factor propeptide fused to the
20 58 amino acid sequence of placental bikunin (102-159). Processing of this fusion protein at a KEX-2 cleavage site at the junction between the α -mating factor and Kunitz domain was designed to liberate the Kunitz domain at its native N-terminus.

25 A 5' sense oligonucleotide of the following sequence and containing a HindIII site for cloning was synthesized:

GAA GGG GTA AGC TTG GAT AAA AGA TAT GAA GAA TAC TGC ACC
GCC AAC GCA GTC ACT GGG CCT TGC CGT GCA TCC TTC CCA CGC
TGG TAC TTT GAC GTG GAG AGG (SEQ ID NO: 42)

30

A 3' antisense oligonucleotide of the following sequence and containing both a BamHI site for cloning and a stop codon was synthesized:

5 CGC GGA TCC CTA CTG GCG GAA GCA GCG GAG CAT GCA GGC CTC
CTC AGA GCG GTA GCT GTT CTT ATT GCC CCG GCA GCC TCC ATA
GAT GAA GTT ATT GCA GGA GTT CCT CTC CAC GTC AAA GTA CCA
GCG
(SEQ ID NO: 43)

10 The oligonucleotides were dissolved in 10 mM Tris buffer pH 8.0 containing 1 mM EDTA, and 12 ug of each oligo were added combined and brought to 0.25M NaCl. To hybridize, the oligonucleotides were denatured by boiling for 5 minutes and allowed to cool from 65°C to room temp over 2 hrs. Overlaps were extended using the Klenow fragment and digested with HindIII
15 and BamHI. The resulting digested double stranded fragment was cloned into pUC19 and sequence confirmed. A clone containing the fragment of the correct sequence was digested with BamHI/HindIII to liberate the bikunin containing fragment with the following + strand sequence:

20 GAA GGG GTA AGC TTG GAT AAA AGA TAT GAA GAA TAC TGC ACC
GCC AAC GCA GTC ACT GGG CCT TGC CGT GCA TCC TTC CCA CGC
TGG TAC TTT GAC GTG GAG AGG AAC TCC TGC AAT AAC TTC ATC
TAT GGA GGC TGC CGG GGC AAT AAG AAC AGC TAC CGC TCT GAG
25 GAG GCC TGC ATG CTC CGC TGC TTC CGC CAG TAG GGA TCC (SEQ
ID. : 44)

which was then gel purified and ligated into BamHI/HindIII cut pS604. The ligation mixture was extracted into phenol/chloroform and purified over a S-200 minispin column. The ligation product was directed transformed into yeast
30 strains SC101 and WHL341 and plated on ura selection plates. Twelve colonies from each strain were re-streaked on ura drop out plates. A single colony was inoculated into 2 ml of ura DO media and grown over night at 30°C. Cells were pelleted for 2 minutes at 14000x g and the supernatants evaluated for their content of placental bikunin (102-159).

35 *Detection of expression of placental bikunin (102-159) in transformed yeast*

Firstly, the supernatants (50 ul per assay) were evaluated for their capacity to inhibit the *in vitro* activity of trypsin using the assay methods as described in Example 1 (1 ml assay volume). An un-used media only sample as
40 well as a yeast clone expressing an inactive variant of aprotinin served as

negative controls. A yeast clone expressing natural aprotinin served as a positive control and is shown for comparison.

The second method to quantify placental bikunin (102-159) expression exploited use of polyclonal antibodies (pAbs) against the synthetic peptide to monitor the accumulation of the recombinant peptide using Western blots. These studies were performed only with recombinants derived from strain SC101, since these produced greater inhibitory activity than recombinants derived from strain WHL341.

To produce the pAb, two 6-8 week old New Zealand White female rabbits (Hazelton Research Labs, Denver, Pa) were immunized on day zero with 250 ug of purified reduced synthetic placental bikunin (102-159), in Complete Freund's adjuvant, followed by boosts on days 14, 35 and 56 and 77 each with 125 ug of the same antigen in Incomplete Freund's adjuvant. Antiserum used in the present studies was collected after the third boost by established procedures. Polyclonal antibodies were purified from the antiserum over protein A.

Colonies 2.4 and 2.5 from transformation of yeast SC101 (Figure 8) as well as an aprotinin control were grown overnight in 50 ml of ura DO media at 30°C. Cells were pelleted and the supernatant concentrated 100-fold using a Centriprep 3 (Amicon, Beverly, MA) concentrator. Samples of each (30 µl) were subjected to SDS-PAGE on 10-20% tricine buffered gels (Novex, San Diego, CA) using the manufacturers procedures. Duplicate gels were either developed with a silver stain kit (Integrated Separation Systems, Nantick, MA) or transferred to nitrocellulose and developed with the purified polyclonal antibody elicited to synthetic bikunin (102-159). Alkaline-phosphatase conjugated goat anti-rabbit antibody was used as the secondary antibody according to the manufacturer's directions (Kirkegaard and Perry, Gaithersburg, MD).

Purification of placental bikunin (102-159) from a transformed strain of SC101

Fermentation broth from a 1L culture of SC101 strain 2.4 was harvested by centrifugation (4,000 g x 30 min.) then applied to a 1.0 ml column of anhydrochymotrypsin-sepharose (Takara Biochemical Inc., CA), that was previously equilibrated with 50 mM Hepes buffer pH 7.5 containing 0.1M NaCl, 2 mM CaCl₂ and 0.01% (v/v) triton X-100. The column was washed with the same buffer but containing 1.0 M NaCl until the A280nm declined to zero, whereupon the column was eluted with 0.1M formic acid pH 2.5. Eluted fractions were pooled and applied to a C18 column (Vydac, 5µm, 4.6 x 250 mm)

previously equilibrated with 0.1% TFA, and eluted with a 50 min. linear gradient of 20 to 80% acetonitrile in 0.1% TFA. Fractions containing placental bikunin (102-159) were pooled and re-chromatographed on C18 employing elution with a linear 22.5 to 50% acetonitrile gradient in 0.1% TFA.

Results. Figure 8 shows the percent trypsin activity inhibited by twelve colonies derived from the transformation of each of strains SC101 and WHL341. The results show that all twelve colonies of yeast strain SC101 transformed with the trypsin inhibitor placental bikunin (102-159) had the ability to produce a substantial amount of trypsin inhibitory activity compared to the negative controls both of which showed no ability to inhibit trypsin. The activity is therefore related to the expression of a specific inhibitor in the placental bikunin variant (102-159) transformed cells. The yeast WHL341 samples contained minimal trypsin inhibitory activity. This may be correlated to the slow growth observed with this strain under the conditions employed.

Figure 9 shows the SDS-PAGE and western analysis of the yeast SC101 supernatants. Silver stained SDS-PAGE of supernatants derived from recombinant yeasts 2.4 and 2.5 expressing placental bikunin (102-159) as well as from the yeast expressing aprotinin yielded a protein band running at approximated 6 kDa, corresponding to the size expected for each recombinant Kunitz inhibitor domain. Western analysis showed that the 6 kDa bands expressed by stains 2.4. and 2.5 reacted with the pAb elicited to placental bikunin (102-159). The same 6 kDa band in the aprotinin control did not react with the same antibody, demonstrating the specificity of the antibody for the placental bikunin variant (102-159).

The final preparation of placental bikunin C-terminal domain was highly pure by silver-stained SDS-PAGE (Figure 10). The overall recovery of broth-derived trypsin inhibitory activity in the final preparation was 31%. N-terminal sequencing of the purified inhibitor indicated that 40% of the protein is correctly processed to yield the correct N-terminus for placental bikunin (102-159) while about 60 % of the material contained a portion of the yeast α -mating factor. The purified material comprised an active serine protease inhibitor exhibiting an apparent K_i of 0.35 nM for the *in vitro* inhibition of plasma kallikrein.

In conclusion, the accumulation both of a protease inhibitor activity and a protein immunochemically related to synthetic bikunin (102-159) in fermentation broth as well as the isolation of placental bikunin (102-159) from

one of the transformed lines provided proof of expression of placental bikunin in the recombinant yeast strains described herein, showing for the first time the utility of yeasts for the production of placental bikunin fragments.

Additional constructs were prepared in an effort to augment the expression level of the Kunitz domain contained within placental bikunin 102-159, as well as to increase the yield of protein with the correct N-terminus. We hypothesized that the N-terminal residues of placental bikunin 102-159 (YEEY--) may have presented a cleavage site that is only poorly recognized by the yeast KEX-2 protease that enzymically removes the yeast a-factor pro-region. Therefore, we prepared yeast expression constructs for the production of placental bikunin 103-159 (N-terminus of EEY...), 101-159 (N-terminus of NYEEY...) and 98-159 (DMFNYEEY..) in order to modify the P' subsites surrounding the KEX-2 cleavage site. To attempt to augment the levels of recombinant protein expression, we also used the yeast preferred codons rather than mammalian preferred codons in preparing some of the constructs described below. The constructs were essentially prepared as described above for placental bikunin 102-159 (defined as construct #1) but with the following modifications:

Construct #2 placental bikunin 103-159, yeast codon usage
A 5' sense oligonucleotide

GAAGGGGTAA GCTTGGATAA AAGAGAAGAA TACTGTACTG
CTAATGCTGT TACTGGTCCA TGTAGAGCTT CTTTCCAAG
ATGGTACTTT GATGTTGAAA GA (SEQ ID NO: 55)

and 3' antisense oligonucleotide

ACTGGATCCT CATTGGCGAA AACATCTCAA CATAcAGGCT
TCTTCAGATC TGTAAGAATT TTTATTACCT CTACAACCAC
CGTAAATAAA ATTATTACAA GAATTTCCTT CAACATCAAA
GTACCATCT (SEQ ID NO: 56)

were manipulated as described for the production of an expression construct (construct #1 above) for the expression of placental bikunin 102-159

Construct #3 placental bikunin 101-159, yeast codon usage

A 5' sense oligonucleotide

GAAGGGGTAA GCTTGGATAA AAGAAATTAC GAAGAATACT
GTACTGCTAA TGCTGTTACT GGTCCATGTA GAGCTTCTTT
5 TCCAAGATGG TACTTTGATG TTGAAAGA (SEQ ID NO: 57)

and the same 3' antisense oligonucleotide as used for construct #2, were
manipulated as described for the production of an expression construct
(construct #1 above) for the expression of placental bikunin 102-159.

10

Construct #4 placental bikunin 98-159, yeast codon usage
A 5' sense oligonucleotide

GAAGGGGTAA GCTTGGATAA AAGAGATATG TTTAATTACG
15 AAGAATACTG TACTGCTAAT GCTGTTACTG GTCCATGTAG
AGCTTCTTTT CCAAGATGGT ACTTTGATGT TGAAAGA (SEQ ID NO: 58)

and the same 3' antisense oligonucleotide as used for construct #2, were
manipulated as described for the production of an expression construct
20 (construct #1 above).

Yeast strain SC101 (MAT α , ura 3-52, suc 2) was transformed with the
plasmids containing each of the above cDNAs, and proteins were expressed
using the methods that were described above for the production of placental
bikunin 102-159 with human codon usage. Approximately 250 ml of each yeast
25 culture was harvested, and the supernatant from centrifugation (15 min x 3000
RPM) separately subjected to purification over 1 ml columns of kallikrein-
sepharose as described above. The relative amount of trypsin inhibitory activity
in the aplysate, the amount of purified protein recovered and the N-terminal
sequence of the purified protein were determined and are listed below in Table
30 7.

Table 7
Relative production levels of different proteins containing the C-terminal Kunitz domain of placental bikunin

| TABLE 7 | | | | | |
|-----------|---|---|--------------------------------------|----------|--|
| Construct | | Relative conc. of inhibitor in aplysate | N-terminal sequencing: amount (pmol) | sequence | Comments |
| 10 | #2 103-159 | none detected | none | none | no expression |
| | #3 101-159 | 25 % inhibition | none | none | low expression |
| 15 | #4 98-159 expression correct product | 93 % inhibition | 910 | DMFNYE- | good |
| 20 | #1 102-159 | 82 % inhibition | 480 | AKEEGV- | expression of active incorrectly processed protein |

The results show that placental bikunin fragments of different lengths that contain the C-terminal Kunitz domain show wide variation in capacity to express functional secreted protein. Constructs expressing fragments 101-159 and 103-159 yielded little or low enzymic activity in the supernatants prior to purification, and N-terminal sequencing of 0.05 ml aliquots of each purified fraction yielded undetectable amounts of inhibitor. On the other hand expression either of placental bikunin 102-159 or 98-159 yielded significant amounts of protease activity prior to purification. N-terminal sequencing however showed that the purified protein recovered from expression of 102-159 was once again largely incorrectly processed, exhibiting an N-terminus consistent with processing of the majority of the pre-protein at a site within the yeast α -mating factor pro-sequence. The purified protein recovered from expression of placental bikunin 98-159 however was processed entirely at the correct site to yield the correct N-terminus. Furthermore, nearly twice as much protein was recovered as compared to the recovery of placental bikunin 102-159. Placental bikunin 98-159 thus represents a preferred fragment length for the production of the C-terminal Kunitz domain of placental bikunin by the α -mating factor pre-pro sequence/ KEX-2 processing system of *S. cerevisiae*,

Example 6**Alternative procedure for yeast expression**

The 58 amino acid peptide derived from the R74593 translation product can also be PCR amplified from either the R87894-R74593 PCR product cloned into the TA vector™ (Invitrogen, San Diego, CA) after DNA sequencing or from human placental cDNA. The amplified DNA product will consist of 19 nucleotides from the yeast α -mating factor leader sequence mated to the R74593 sequence which codes for the YEEY--CFRQ (58 residues) so as to make the translation product in frame, constructing an α -mating factor/Kunitz domain fusion protein. The protein sequence also contains a kex 2 cleavage which will liberate the Kunitz domain at its native N-terminus.

The 5' sense oligonucleotide which contains a HindIII site for cloning will contain the following sequence:

15 GCCAAGCTTG GATAAAAGAT ATGAAGAAT ACTGCACCGC CAACGCA
(SEQ ID NO: 30)

The 3' antisense oligonucleotide contains a BamHI site for cloning as well as a stop codon and is of the following sequence:

20 GGGGATCCTC ACTGCTGGCG GAAGCAGCGG AGCAT (SEQ ID NO: 31)

The full 206 nucleotide cDNA sequence to be cloned into the yeast expression vector is of the following sequence:

25 CCAAGCTTGG ATAAAAGATA TGAAGAATAC TGCACCGCCA ACGCAGTCAC
TGGGCCTTGC CGTGCATCCT TCCCACGCTG GTACTTTGAC GTGGAGAGGA
ACTCCTGCAA TAAC TTCATC TATGGAGGCT GCCGGGGCAA TAAGAACAGC
TACCGCTCTG AGGAGGCCTG CATGCTCCGC TGCTTCCGCC AGCAGTGAGG
ATCCCC (SEQ ID NO: 32)

30

After PCR amplification, this DNA will be digested with HindIII, BamHI and cloned into the yeast expression vector pMT15 (see US patent 5,164,482, incorporated by reference in the entirety) also digested with HindIII and BamHI. The resulting plasmid vector is used to transform yeast strain SC 106 using the methods described in US patent 5,164,482. The URA 3+ yeast transformants are isolated and cultivated under inducing conditions. The yield of recombinant Placental bikunin variants is determined according to the

35

amount of trypsin inhibitory activity that accumulated in the culture supernatants over time using the *in vitro* assay method described above. Fermentation broths are centrifuged at 9000 rpm for 30 minutes. The supernatant is then filtered through a 0.4 then a 0.2 μ m filter, diluted to a conductivity of 7.5 ms, and adjusted to pH 3 with citric acid. The sample is then batch absorbed onto 200 ml of S-sepharose fast flow (Pharmacia) in 50 mM sodium citrate pH 3 and stirred for 60 min. The gel is subsequently washed sequentially with 2 L of each of: 50 mM sodium citrate pH 3.0; 50 mM Tris-HCL pH 9.0; 20 mM HEPES pH 6.0. The washed gel is transferred into a suitable column and eluted with a linear gradient of 0 to 1 M sodium chloride in 20 mM HEPES pH 6.0. Eluted fractions containing *in vitro* trypsin inhibitory activity are then pooled and further purified either by a) chromatography over a column of immobilized anhydrotrypsin (essentially as described in Example 2); b) by chromatography over a column of immobilized bovine kallikrein; or c) a combination of conventional chromatographic steps including gel filtration and/or anion-exchange chromatography.

Example 7

Isolation and characterization of native human placental bikunin from placenta

Bikunin protein was purified to apparent homogeneity from whole frozen placenta (Analytical Biological Services, Inc, Wilmington, DE). The placenta (740 gm) was thawed to room temperature and cut into 0.5 to 1.0 cm pieces, placed on ice and washed with 600 ml PBS buffer. The wash was decanted and 240 ml of placenta pieces placed into a Waring blender. After adding 300 ml of buffer consisting of 0.1 M Tris (pH 8.0), and 0.1 M NaCl, the mixture was blended on high speed for 2 min, decanted into 750.0 ml centrifuge tubes, and placed on ice. This procedure was repeated until all material was processed. The combined slurry was centrifuged at 4500 x g for 60 minutes at 4°C. The supernatant was filtered through cheese cloth and the placental bikunin purified using a kallikrein affinity column made by covalently attaching 70 mg of bovine pancreatic kallikrein (Bayer AG) to 5.0 mls of CNBr activated Sepharose (Pharmacia) according to manufacturers instruction. The material was loaded onto the affinity column at a flow rate of 2.0 ml/min and washed with 0.1 M Tris (pH 8.0), 0.1 M NaCl until absorbance at 280 nm of the wash could no longer be detected. The column was further washed with 0.1 M Tris (pH 8.0), 0.5 M NaCl and then eluted with 3 volumes of 0.2 M acetic acid,

pH 4.0. Fractions containing kallikrein and trypsin inhibitory (see below) activity were pooled, frozen, and lyophilized. Placental bikunin was further purified by gel-filtration chromatography using a Superdex 75 10/30 (Pharmacia) column attached to a Beckman System Gold HPLC system. Briefly, the column was equilibrated in 0.1 M Tris, 0.15 M NaCl, and 0.1% Triton X-100 at a flow rate of 0.5 ml/min. The lyophilized sample was reconstituted in 1.0 ml of 0.1 M Tris, pH 8.0 and injected onto the gel-filtration column in 200 µl aliquots. Fractions were collected (0.5 ml) and assayed for trypsin and kallikrein inhibitory activity. Active fractions were pooled, and the pH of the solution adjusted to 2.5 by addition of TFA. The material was directly applied to a Vydac C18 reverse-phase column (5 micron, 0.46 x 25 cm) which had been equilibrated in 20% acetonitrile in 0.1 %TFA. Separation was achieved using a linear gradient of 20 to 80% acetonitrile in 0.1% TFA at 1.0 ml/min over 50 minutes after an initial 20 minute wash at 20% acetonitrile in 0.1% TFA. Fractions (1ml) were collected and assayed for trypsin and kallikrein inhibitory activity. Fractions containing inhibitory activity were concentrated using a speed-vac concentrator (Savant) and subjected to N-terminal sequence analysis.

Functional assays for Placental Bikunin:

Identification of functional placental bikunin was achieved by measuring its ability to inhibit bovine trypsin and human plasma kallikrein. Trypsin inhibitory activity was performed in assay buffer (50 mM Hepes, pH 7.5, 0.1 M NaCl, 2.0 mM CaCl₂, 0.1% Triton x-100) at room temperature in a 96-well microtiter plate (Perkin Elmer) using Gly-Pro-Lys-Aminomethylcoumarin as a substrate. The amount of coumarin produced by trypsin was determined by measuring the fluorescence (ex = 370 nm, em = 432 nm) on a Perkin-Elmer LS-50B fluorimeter equipped with a plate reader. Trypsin (23 µg in 100 µl buffer) was mixed with 20 µl of the sample to be tested and incubated for 10 minutes at 25°C. The reaction was started by the addition of 50 µl of the substrate GPK-AMC (33 µM final) in assay buffer. The fluorescence intensity was measured and the % inhibition for each fraction was determined by:

$$\% \text{ inhibition} = 100 \times [1 - F_0/F_1]$$

where F_0 is the fluorescence of the unknown and F_1 is the fluorescence of the trypsin only control. Kallikrein inhibitory activity of the fractions was similarly measured using 7.0 nM kallikrein in assay buffer (50 mM Tris, pH 8.0, 50 mM

NaCl, 0.1% triton x-100) and 66.0 μ M Pro-Phe-Arg-AMC as a substrate.

Determination of the *in vitro* specificity of placental bikunin

The *In vitro* specificity of native human placental bikunin was determined using the materials and methods as described in the preceding examples above. Placental bikunin was quantified by active site titration against a known concentration of trypsin using GPK-AMC as a substrate to monitor the fraction of unbound trypsin.

Protein Sequencing

The 1 ml fraction (C18-29 Delaria) was reduced to 300 ml in volume, on a Speed Vac, to reduce the amount of organic solvent. The sample was then loaded onto a Hewlett-Packard miniature biphasic reaction column, and washed with 1 ml of 2% trifluoroacetic acid. The sample was sequenced on a Hewlett-Packard Model G1005A protein sequencing system using Edman degradation. Version 3.0 sequencing methods and all reagents were supplied by Hewlett-Packard. Sequence was confirmed for 50 cycles.

Results. Placental Bikunin was purified to apparent homogeneity by sequential kallikrein affinity, gel-filtration, and reverse-phase chromatography (see purification table below):

Table 5
Purification table for native Placental Bikunin (1-179)

| TABLE 5 | | | | | | |
|----------------------------|----------|--------------|--------|------------------------|--------------|--|
| Step | Vol (ml) | OD 280 (/ml) | OD 280 | Units ^a (U) | Units/OD 280 | |
| Placenta Supernatant | 1800.0 | 41.7 | 75,060 | 3,000,000 | 40.0 | |
| Kallikrein Affinity pH 4.0 | 20.0 | 0.17 | 3.36 | 16,000 | 4,880 | |
| Kallikrein Affinity pH 1.7 | 10.2 | 0.45 | 4.56 | 12,000 | 2,630 | |
| Superdex 75 | 15.0 | 0.0085 | 0.13 | 3,191 | 24,546 | |

^aOne Unit is defined as that amount which inhibits 50% of trypsin activity in a standard assay.

The majority of the kallikrein and trypsin inhibitory activity eluted from the kallikrein affinity column in the pH 4.0 elution. Subsequent gel-filtration chromatography (Figure 5) yielded a peak of kallikrein and trypsin inhibitory

activity with a molecular weight range of 10 to 40 kDa as judged by a standard curve generated by running molecular weight standards under identical conditions. Reverse-phase C18 chromatography (Figure 6) yielded 4 peaks of inhibitory activity with the most potent eluting at approximately 30 % acetonitrile. The activity associated with the first peak to elute from C18 (fraction 29) exhibited an amino acid sequence starting with amino acid 1 of the predicted amino acid sequence of placental bikunin (ADRER...; SEQ ID NO: 1), and was identical to the predicted sequence for 50 cycles of sequencing (underlined amino acids in Figure 3). Cysteine residues within this sequence stretch were silent as expected for sequencing of oxidized protein. The cysteine residues at amino acid positions 11 and 20 of mature placental bikunin were later identified from sequencing of the S-pyridylethylated protein whereupon PTH-pyridylethyl-cysteine was recovered at cycles 11 and 20.

Interestingly, the asparagine at amino acid residue number 30 of the sequence (Figure 3) was silent showing that this site is likely to be glycosylated. Fraction 29 yielded one major sequence corresponding to that of placental bikunin starting at residue #1 (27 pmol at cycle 1) plus a minor sequence (2 pmol) also derived from placental bikunin starting at residue 6 (SIHD...). This shows that the final preparation sequenced in fraction 29 is highly pure, and most likely responsible for the protease inhibitory activity associated with this fraction (Figure 6).

Accordingly, the final preparation of placental bikunin from C18 chromatography was highly pure based on a silver-stained SDS-PAGE analysis (Figure 7), where the protein migrated with an apparent Mr of 24 kDa on a 10 to 20 % acrylamide tricine gel (Novex, San Diego, CA) calibrated with the following molecular weight markers: insulin (2.9 kDa); bovine trypsin inhibitor (5.8 kDa); lysozyme (14.7 kDa); β -lactaglobulin (18.4 kDa); carbonic anhydrase (29 kDa); and ovalbumin (43 kDa). The above size of placental bikunin on SDS-PAGE is consistent with that predicted from the full length coding sequence (Figure 4F).

As expected based on the N-terminal sequencing results described above, the purified protein reacted with an antibody elicited to placental bikunin (7-64) to yield a band with the same Mr (Figure 12A) as observed for the purified preparation detected on gels by silver stain (Figure 7). However, when the same preparation was reacted with an antibody elicited to synthetic placental bikunin (102-159), a band corresponding to the full length protein was not observed. Rather, a fragment that co-migrated with synthetic bikunin (102-

159) of approximately 6 kDa was observed. The simplest interpretation of these results is that the purified preparation had undergone degradation subsequent to purification to yield an N-terminal fragment comprising the N-terminal domain and a C-terminal fragment comprising the C-terminal domain. Assuming that the fragment reactive against antiserum to placental bikunin (7-64) is devoid of the C-terminal end of the full length protein, the size (24 kDa) would suggest a high state of glycosylation.

Table 6. below shows the potency of *in vitro* inhibition of various serine proteases by placental bikunin. Data are compared with that obtained with aprotinin (Trasylo[®]).

Table 6
Ki values for the inhibition of various proteases by placental bikunin

| TABLE 6 | | |
|-----------------------------|------------------------------|----------------------|
| Protease (concentration) | Placental Bikunin Ki (nM) | Aprotinin Ki (nM) |
| Trypsin (48.5 pM) | 0.13 | 0.8 |
| Human Plasmin (50 pM) | 1.9 | 1.3 |

The results show that placental bikunin isolated from a natural source (human placenta) is a potent inhibitor of trypsin-like serine proteases.

Example 8

20 Expression pattern of placental bikunin amongst different human organs and tissues

A multiple tissue northern was purchased from Clontech which contained 2 µg of polyA⁺ RNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. Two different cDNA probes were used: 1) a gel purified cDNA encoding placental bikunin (102-159); 2) the 780 base pair PCR-derived cDNA (Figure 4E) liberated from a TA clone by digestion with EcoRI and gel purified. Each probe was labeled using ³²P-dCTP and a random priming labeling kit from Boehringer Mannheim Biochemicals (Indiana), then used to hybridize to the multiple tissue northern according to the manufacturers specifications. Autoradiographs were generated using Biomax film with an 18 hr exposure time, and developed using a Umax Scanner and scanned using Adobe Photoshop.

Results. The pattern of tissue expression observed using a placental bikunin (102-159) probe (Figure 11A) or a larger probe containing both Kunitz domains of placental bikunin (Figure 11B) was essentially the same as might be expected. The placental bikunin mRNA was most abundant in pancreas and placenta. Significant levels were also observed in lung, brain and kidney, while lower levels were observed in heart and liver, and the mRNA was undetectable in skeletal muscle. The transcript size was 1.95 kilobases in all cases, in close agreement with the predicted size of placental bikunin deduced both from EST overlay and cloning of full length cDNA described in preceding sections.

The broad tissue distribution of the mRNA shows that placental bikunin is broadly expressed. Since the protein also contains a leader sequence it would have ample exposure to the human immune system, requiring that it become recognized as a self protein. Additional evidence for a broad tissue distribution of placental bikunin mRNA expression was derived from the fact that some of the EST entries with homology to placental bikunin (Figure 4B) were derived from human adult and infant brain, and human retina, breast, ovary, olfactory epithelium, and placenta. It is concluded therefore that administration of the native human protein to human patients would be unlikely to elicit an immune response.

Interestingly, the expression pattern of placental bikunin is somewhat reminiscent of that for bovine aprotinin which is found in high levels in bovine lung and pancreas. To further elucidate the expression pattern of placental bikunin, RT-PCR of total RNA from the following human cells was determined: un-stimulated human umbilical vein endothelial cells (HUVECs), HK-2 (line derived from kidney proximal tubule), TF-1 (erythroleukemia line) and phorbol ester (PMA)-stimulated human peripheral blood leukocytes. The probes used:

CACCTGATCGCGAGACCCC (sense; SEQ ID NO: 59);

CTGGCGGAAGCAGCGGAGCATGC (antisense; SEQ ID NO: 60),

were designed to amplify a 600 b.p placental bikunin encoding cDNA fragment. Comparisons were normalized by inclusion of actin primers to amplify an 800 b.p. actin fragment. Whereas the 800 b.p fragment identified on agarose gels with ethidium bromide was of equal intensity in all lanes, the 600 b.p. placental bikunin fragment was absent from the HUVECs but present in significant amounts in each of the other cell lines. We conclude that placental

bikunin is not expressed in at least some endothelial cells but is expressed in some leukocyte populations.

Example 9

5 Purification and properties of Placental Bikunin (1-170) highly purified from a Baculovirus / Sf9 expression system

A large fragment of Placental bikunin containing both Kunitz domains (Placental Bikunin 1-170) was expressed in Sf9 cells as follows. Placental bikunin cDNA obtained by PCR (Figure 4E) and contained within a TA vector
 10 (see previous Examples) was liberated by digestion with HindIII and XbaI yielding a fragment flanked by a 5' XbaI site and 3' HindIII site. This fragment was gel purified and then cloned into the M13mp19 vector (New England Biolabs, Beverly, MA). In vitro mutagenesis (Kunkel T.A., (1985) Proc. Natl. Acad. Sci. USA, 82: 488-492) was used to generate a PstI site 3' to the XbaI site
 15 at the 5' end, but 5' to the sequence encoding the ATG start site, natural placental bikunin signal peptide and mature placental bikunin coding sequence. The oligonucleotide used for the mutagenesis had the sequence:

20 5' CGC GTC TCG GCT GAC CTG GCC CTG CAG ATG GCG CAC GTG TGC GGG 3' (SEQ ID NO: 61)

A stop codon (TAG) and BglII / XmaI site was similarly engineered at the 3' end of the cDNA using the oligonucleotide:

25 5' CTG CCC CTT GGC TCA AAG TAG GAA GAT CTT CCC CCC GGG GGG GTG GTT CTG GCG GGG CTG 3' (SEQ ID NO: 62).

The stop codon was in frame with the sequence encoding placental bikunin and caused termination immediately following the Lysine at amino acid residue
 30 170, thus encoding a truncated placental bikunin fragment devoid of the putative transmembrane domain. The product from digestion with PstI and BglII was isolated and cloned into the BacPac8 vector for expression of Placental bikunin fragment (1-170) which contains both Kunitz domains but which is truncated immediately N-terminal to the putative transmembrane
 35 segment.

The expression of Bikunin by Sf-9 insect cells was optimal at a multiplicity of infection of 1 to 1 when the medium was harvested at 72 h post

infection. After harvesting, the baculovirus cell culture supernatant (2L) was adjusted to pH 8.0 by the addition of Tris-HCl. Bikunin was purified by chromatography using a 5 ml bovine pancreatic kallikrein affinity column as previously described in Example 7 for the purification of native placental bikunin from placenta. Eluted material was adjusted to pH 2.5 with TFA and subjected to chromatography on a C18 reverse-phase column (1.0 x 25 cm) equilibrated in 10% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min. The bikunin was eluted with a linear gradient of 10 to 80% acetonitrile in 0.1% TFA over 40 min. Active fractions were pooled, lyophilized, redissolved in 50 mM Hepes (pH 7.5), 0.1 M NaCl, 2 mM CaCl₂, and 0.1% triton x-100, and stored at -20°C until needed. The concentration of recombinant bikunin was determined by amino acid analysis.

Results. Recombinant bikunin was purified from baculovirus cell culture supernatant using a 2-step purification protocol as shown below, to yield an active trypsin inhibitor (Table 8 below).

Table 8
Purification of recombinant bikunin from transformed culture supernatant

TABLE 8

| Purification Step | Vol (ml) | OD 280/ml | OD 280 total | Units (U) | Specific activity (U/OD) |
|---------------------|----------|-----------|--------------|-----------|--------------------------|
| Supernatant | 2300.0 | 9.0 | 20,700 | 6,150,000 | 297 |
| Kallikrein affinity | 23.0 | 0.12 | 2.76 | 40,700 | 14,746 |
| C18 reverse-phase | 0.4 | 3.84 | 1.54 | 11,111 | 72,150 |

Chromatography of the crude material over an immobilized bovine pancreatic kallikrein affinity column selectively isolated 0.013 % of the protein and 0.67 % of the trypsin inhibitory activity present. The majority of the trypsin inhibitory activity present in the starting supernatant did not bind to the immobilized kallikrein and is not related to bikunin (results not shown). Subsequent chromatography using C18 reverse-phase yielded a further purification of 5-fold, with a recovery of 0.2%. The final preparation was highly pure by SDS-PAGE (Figure 13), exhibiting an Mr of 21.3 kDa, and reacted on immunoblots to rabbit anti-placental bikunin 102-159 (not shown). N-terminal sequencing (26 cycles) yielded the expected sequence for mature placental bikunin (Figure 4F) starting at residue +1(ADRER....), showing that the signal

peptide was correctly processed in Sf9 cells.

Purified placental bikunin from Sf9 cells (100 pmol) was pyridylethyl-alkylated, CNBr digested and then sequenced without resolution of the resulting fragments. Sequencing for 20 cycles yielded the following N-terminii:

5

| Sequence | Amount | Placental bikunin residue # |
|-------------------------|---------|-----------------------------|
| LRCFrQQENPP-PLG----- | 21 pmol | 154 - 168 (SEQ ID NO: 63) |
| ADRErSIHDFCLVSKVVGRC | 20 pmol | 1 - 20 (SEQ ID NO: 64) |
| 10 FNYeEYCTANAVTGPCRASf | 16 pmol | 100 - 119 (SEQ ID NO: 65) |
| Pr--Y-V-dGS-Q-F-Y-G | 6 pmol | 25 - 43 (SEQ ID NO: 66) |

Thus N-terminii corresponding to each of the expected four fragments were recovered. This confirms that the Sf9 expressed protein contained the entire ectodomain sequence of placental bikunin (1-170). N-terminal sequencing (50 cycles) of an additional sample of undigested Placental Bikunin (1-170) resulted in an amino acid sequence which at cycle 30 was devoid of any PTH-amino acid (PTH-asparagine was expected). A similar result was obtained upon sequencing of the natural protein from human placenta (Example 7) and is consistent with this residue being glycosylated as predicted from the amino acid sequence surrounding this asparagine residue. Furthermore, the cysteine residues within this region were also silent consistent with their participation in disulfide bonding.

25 Example 10

Inhibition specificity of purified placental bikunin derived from Sf9 cells.

The *in vitro* specificity of recombinant bikunin was determined using the materials and methods as described in Examples 3, 4 and 7. In addition, the inhibition of human tissue kallikrein by bikunin was measured by the incubation of 0.35 nM human tissue kallikrein recombinant bikunin in buffer containing 50 mM Tris (pH 9.0), 50 mM NaCl, and 0.01% triton x-100. After 5 min. at 37°C, 5 µl of 2 mM PFR-AMC was added and the change in fluorescence monitored.

Inhibition of tissue plasminogen activator (tPA) was also determined as follows: tPA (single chain form from human melanoma cell culture from Sigma Chemical Co, St Louis, MO) was pre-incubated with inhibitor for 2 hr at room temperature in 20 mM Tris buffer pH 7.2 containing 150 mM NaCl, and 0.02% sodium azide. Reactions were subsequently initiated by transfer to a reaction

system comprising the following initial component concentrations: tPA (7.5 nM), inhibitor 0 to 6.6 μ M, Dile-Lpro-Larg-pNitroaniline (1mM) in 28 mM Tris buffer pH 8.5 containing 0.004 % (v/v) triton x-100 and 0.005% (v/v) sodium azide. Formation of p-Nitroaniline was determined from the A405nm measured following incubation at 37 C for 2hr.

The table below show the efficacy of recombinant bikunin as an inhibitor of various serine proteases *in vitro*. Data is shown compared against data obtained for screening inhibition using either recombinant bikunin, or aprotinin.

Table 9
Comparisons of Ki values for the inhibition of various proteases by recombinant placental bikunin (1-170) or aprotinin

| TABLE 9 | | |
|--|--------------------------------|------------------------------|
| Protease (concentration) | Recombinant Bikunin Ki (nM) | Aprotinin Ki (nM) |
| Trypsin (48.5 pM) | 0.064 | 0.8 |
| Human Plasma Kallikrein (2.5 nM) | 0.18 | 19.0 |
| Human Tissue Kallikrein (0.35 nM) | 0.04 | 0.004 |
| Bovine Pancreatic Kallikrein (100 pM) | 0.12 | 0.02 |
| Human Plasmin (50 pM) | 0.23 | 1.3 |
| factor Xa (0.87 nM) | 180 | 5% Inhibition at 31 μ M |
| factor XIa (0.1 nM) | 3.0 | 288 |
| tissue plasminogen activator (7.5 nM) | < 60 | no inhibition at 6.6 μ M |
| Tissue Factor VIIa | 800 | no inhibition at 1 μ M |

The results show that recombinant bikunin can be expressed in insect cells to yield an active protease inhibitor that is effective against at least five different serine protease inhibitors. Recombinant bikunin was more potent than aprotinin against human plasma kallikrein, trypsin and plasmin. Surprisingly, the recombinant bikunin was more potent than the synthetically derived bikunin fragments (7-64) and (102-159) against all enzymes tested. These data show that recombinant bikunin is more effective than aprotinin, using *in vitro* assays, and that one would expect better *in vivo* potency.

Besides measuring the potencies against specific proteases, the capacity of placental bikunin (1-170) to prolong the activated partial thromboplastin time (APTT) was evaluated and compared with the activity associated with

aprotinin. Inhibitor was diluted in 20 mM Tris buffer pH 7.2 containing 150 mM NaCl and 0.02% sodium azide and added (0.1 ml) to a cuvette contained within an MLA Electra^R 800 Automatic Coagulation Timer coagulometer (Medical Laboratory Automation, Inc., Pleasantville, N.Y.). The instrument was
5 set to APTT mode with a 300 sec. activation time and the duplicate mode. Following addition of 0.1 ml of plasma (Specialty Assayed Reference Plasma lot 1-6-5185, Helena Laboratories, Beaumont, TX), the APTT reagent (Automated APTT-lot 102345, from Organon Teknika Corp., Durhan, NC) and 25 mM CaCl₂ were automatically dispensed to initiate clotting, and the clotting time
10 was monitored automatically. The results (Figure 14) showed that a doubling of the clotting time required approximately 2 μ M final aprotinin, but only 0.3 μ M Sf9 derived placental bikunin. These data show that placental bikunin is an effective anticoagulant, and usefull as a medicament for diseases involving pathologic activation of the intrinsic pathway of coagulation.

15 Although certain embodiments of the invention have been described in detail for the purpose of illustration, it will be readily apparent to those skilled in the art that the methods and formulations described herein may be modified without departing from the spirit and scope of the invention. Accordingly, the
20 invention is not limited except as by the appended claims.

WE CLAIM:

1. A substantially purified protein, having serine protease inhibitory activity, selected from the group of proteins consisting of materials each of which comprises one of the following amino acid sequences, the amino acids of said sequences being numbered in accordance with the amino acid sequence of native human placental bikunin shown in figure 4F in which the N-terminal residue generated by removal of signal peptide is designated as residue 1:

10 ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 50
 YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
 NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
 ACMLRCFRQQ ENPPLPLGSK 170
 (SEQ ID NO: 52);

15 MAQLCGL RRSRAFLALL GSLLSGVLA -1
 ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 50
 YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
 NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
 20 ACMLRCFRQQ ENPPLPLGSK VVLAGLFVM VLILFLGASM VYLIRVARRN 200
 QERALRTVWS SGDDKEQLVK NTYVL 225
 (SEQ ID NO: 49);

 ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 50
 25 YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
 NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
 ACMLRCFRQQ ENPPLPLGSK VVLAGLFVM VLILFLGASM VYLIRVARRN 200
 QERALRTVWS SGDDKEQLVK NTYVL 225
 (SEQ ID NO: 70);

30 AGSFLAWL GSLLSGVLA -1
 ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 50
 YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
 NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
 35 ACMLRCFRQQ ENPPLPLGSK VVLAGAVS 179
 (SEQ ID NO: 2);

| | | |
|----|--|-----|
| | MLR AEADGVSRLG GLLLLSGVLA | -1 |
| | ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN | 50 |
| | YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF | 100 |
| | NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE | 150 |
| 5 | ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN | 200 |
| | QERALRTVWS SGDDKEQLVK NTYVL | 225 |
| | (SEQ ID NO: 45); | |
| | MAQLCGL RRSRAFLALL GLLLLSGVLA | -1 |
| 10 | ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN | 50 |
| | YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF | 100 |
| | NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE | 150 |
| | ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN | 200 |
| | QERALRTVWS FGD | 213 |
| 15 | (SEQ ID NO: 47); | |
| | ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN | 50 |
| | YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF | 100 |
| | NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE | 150 |
| 20 | ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN | 200 |
| | QERALRTVWS FGD | 213 |
| | (SEQ ID NO: 71); | |
| | IHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN | 50 |
| 25 | YLTKEECLKK CATV | 64 |
| | (SEQ ID NO: 4); | |
| | CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN | 50 |
| | YLTKEECLKK C | 61 |
| 30 | (SEQ ID NO: 5); | |
| | YEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE | 150 |
| | ACMLRCFRQ | 159 |
| | (SEQ ID NO: 6); | |
| 35 | CTANAVTGPC RASFPRWYFD VERNSCNNFI YGGCRGNKNS YRSEE | 150 |
| | ACMLRC | 156 |

(SEQ ID NO: 7);

| | | |
|---|--|-----|
| | IHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN | 50 |
| | YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF | 75 |
| 5 | NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE | 125 |
| | ACMLRCFRQ | 159 |
| | (SEQ ID NO: 3); | |

| | | |
|----|--|-----|
| | CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN | 50 |
| 10 | YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF | 100 |
| | NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE | 150 |
| | ACMLRC | 156 |
| | (SEQ ID NO: 50); | |

| | | |
|----|--|-----|
| 15 | ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN | 25 |
| | YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF | 75 |
| | NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE | 125 |
| | ACMLRCFRQQ ENPPLPLGSK VVVLGAVS | 179 |
| | (SEQ ID NO: 1); and | |

| | | |
|----|---|----|
| 20 | ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN | 50 |
| | YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DS | 92 |
| | (SEQ ID NO: 8). | |

25 2. A protein as in claim 1, wherein said protein is glycosylated, or contains at least one intra-chain cysteine-cysteine disulfide bond, or is both glycosylated and contains at least one intra-chain cysteine-cysteine disulfide bond.

30 3. A pharmaceutical composition for inhibiting serine protease activity, comprising a protein of claim 1 or claim 2 plus a pharmaceutically acceptable carrier.

4. An isolated nucleic acid sequence which encodes for a protein of claim 1.

35 5. A self-replicating protein expression vector containing a nucleic acid sequence which encodes for and is capable of expressing a protein of claim 1 or claim 2.

6. A method for inhibiting serine protease activity comprising contacting serine protease with an effective amount of at least one protein of claim 1 or claim 2.
- 5
7. A method for treating a condition of brain edema, spinal cord edema, multiple sclerosis, ischemia, perioperative blood loss, sepsis, septic shock, fibrosis, disease associated with pathologic blood coagulation or clotting, polytrauma, stroke, cerebral or subarachnoid hemorrhage, inflammation of the brain, inflammation of the spinal cord, cerebral infection, cerebral granulomatosis, spinal infection, spinal granulomatosis, open heart surgery, gastric cancer, cervical cancer, or prevention of metastasis comprising administering to a subject having such a condition an effective amount of the protein of claim 1 or claim 2.
- 10
- 15
8. The method of Claim 7 wherein said condition is brain edema, spinal cord edema, multiple sclerosis, ischemia, perioperative blood loss, sepsis, septic shock, fibrosis, disease associated with pathologic blood coagulation or clotting, stroke, cerebral or subarachnoid hemorrhage, inflammation of the brain, inflammation of the spinal cord, cerebral infection, cerebral granulomatosis, spinal infection, spinal granulomatosis, or open heart surgery.
- 20
9. The method of Claim 7 wherein said condition is gastric cancer, cervical cancer, or prevention of metastasis.
- 25
10. A method for the preparation of a medicament for the treatment of brain edema, spinal cord edema, multiple sclerosis, ischemia, perioperative blood loss, sepsis, septic shock, fibrosis, disease associated with pathologic blood coagulation or clotting, stroke, cerebral or subarachnoid hemorrhage, inflammation of the brain, inflammation of the spinal cord, cerebral infection, cerebral granulomatosis, spinal infection, spinal granulomatosis, open heart surgery, gastric cancer, cervical cancer, or prevention of metastasis.
- 30
11. A method for preparing a protein of claim 1 or claim 2 using recombinant DNA technology.
- 35

FIGURE 1

| | | | | | | |
|--------|-------------|------------|------------|------------|------------|-----|
| R35464 | GGCCGGGTGCG | TTTCTCGCCT | GGCTGGGATC | GCTGCTCCTC | TCTGGGGTCC | 50 |
| ORF | P G R | F S P | G W D R | C S S | L G S | 16 |
| R35464 | TGGCCGGCCG | ACCGAGAACG | CAGCATCCAC | GACTTCTGCC | TGGTGTGCAA | 100 |
| ORF | W P A D | R E R | S I H | D F C L | V S K | 33 |
| R35464 | GGTGGTGGGC | AGATTCCGGG | CCTCCATGCC | TAGGTGGTGG | TACAATGTCA | 150 |
| ORF | V V G | R E R A | S M P | R W W | Y N V T | 50 |
| R35464 | CTGACGGATC | CTGCCAGCTG | TTTGTGTATG | GGGGCTGTGA | CGGAAACAGC | 200 |
| ORF | D G S | C Q L | F V Y G | G C D | G N S | 66 |
| R35464 | AATAATTACC | TGACCAAGGA | GGAGTGCCTC | AAGAAATGTG | CCACTGTAC | 250 |
| ORF | N N Y L | T K E | E C L | K K C A | T V T | 83 |
| R35464 | AGAGAATGCC | ACGGGTGACC | TGGCCACCAG | CAGGAATGCA | GCGGATTCC | 300 |
| ORF | E N A | T G D L | A T S | R N A | A D S S | 100 |
| R35464 | CTGTCCCAAG | TGCTCCCAGA | AGGCAGGATT | CTTGAAGACC | ACTTCAGCGA | 350 |
| ORF | V P S | A P R | R Q D S | * R P | L Q R | 116 |
| R35464 | TATGTTTCAA | NTATTGNAAG | AATAATTGCA | CCGNCAACGN | ATT----- | 393 |
| ORF | Y V S * | I * R | I I A | P * T * | | 130 |

KEY

R35464 = Nucleic acid sequence of EST R35464 (SEQ ID NO: 12)

ORF - EST R35464 Open Reading Frame Translation (SEQ ID NO: 13)

FIGURE 2

```

R74593 GCAATAATTA CCTGACCAAG GAGGAGTGCC TCAAGAAATG TGCCACTGTC 50
ORF    Q * L P D Q G G V P Q E M C H C H 17

R74593 ACAGAGAATG CCACGGGTGA CCTGGCCACC AGCAGGAATG CAGCGGATTC 100
ORF    R E C H G * P G H Q Q E C S G F 33

R74593 CTCTGTCCCA AGTCTCCCAG AAGGCAGGAT TCTGAAGACC ACTCCAGCGA 150
ORF    L C P K S P R R Q D S E D H S S D 50

R74593 TATGTTCAAC TATGAAGAAT ACTGCACCGC CAACGCAGTC ACTGGGCCTT 200
ORF    M F N Y E E Y C T A N A V T G P C 67

R74593 GCCGTGCATC CTTCCCACGC TGGTACTTTG ACGTGGAGAG GAACTCCTGC 250
ORF    R A S F P R W Y F D V E R N S C 83

R74593 AATAACTTCA TCTATGGAGG CTGCCGGGGC AATAAGAACA GCTACCGCTC 300
ORF    N N F I Y G G C R G N K N S Y R S 100

R74593 TGAGGAGGCC TGCATGCTCC GCTGCTTCCG CCAGCAGGAG AATCCTCCCC 350
ORF    E E A C M L R C F R Q Q E N P P L 117

R74593 TGCCCCTTGG CTCAAAGGTG GTGGTTCTGG CCGGGGCTGT TTCGTGATGG 400
ORF    P L G S K V V V L A G A V S * W 133

R74593 TGTGTATCCT TTTCTGGGG AGCNTCCATG GTCTTACTGA TTCCGGGTGG 450
ORF    C * S F S W G A S M V L L I P G G 150

R74593 CAAGGAGGAA CCAGGAGCGT GCCCTGCGGA NCGTCTGGAG CTTCCGAGAT 500
ORF    K E E P G A C P A X R L E L R R * 167

R74593 GACAAGGGNT 510
ORF    Q G 169

```

KEY

R74593 = Nucleic acid sequence of EST R74593 (SEQ ID NO: 14)

ORF = EST R74593 Open Reading Frame Translation (SEQ ID NO: 15)

FIGURE 3.

| | | | | | | |
|--------------|-------------|------------|------------|------------|-------------|-----|
| R35464 | GGCCGGGTCGT | TTCTCGCCTG | GCTGGGA-TC | GCTGCTCCTC | TCTGGGGTCC | 50 |
| N39798 | | | TGGGANTC | GCTGCTCCTC | TCTGGGGTCC | 28 |
| H94519 | GCNCGC-CGT | TNNTCGCNT- | GCTGGGA-TC | GCTGCACCTC | TCTGGGGTCG | 47 |
| R74593 corr. | ----- | ----- | ----- | ----- | ----- | |
| Consensus | GGCCGGGTCGT | TTCTCGCCTG | GCTGGGA-TC | GCTGCTCCTC | TCTGGGGTCC | 50 |
| Translation | A G S F | L A W | L G S | L L L | S G V | -3 |
| R35464 | TGGCCGGCCG | ACCGAGAACG | CAGCATCCAC | GACTTCTGCC | TGGTGTGCGAA | 100 |
| N39798 | TGG-CGGCCG | ACCGAGAACG | CAGCATCCAC | GACTTCTGCC | TGGTGTGCGAA | 77 |
| H94519 | NGG-CGGCCG | ACCGAGAACG | CAGCATCCAC | GACTTCTGCC | TGGTGTGCGAA | 96 |
| R74593 corr. | ----- | ----- | ----- | ----- | ----- | |
| Consensus | TGG-CGGCCG | ACCGAGAACG | CAGCATCCAC | GACTTCTGCC | TGGTGTGCGAA | 99 |
| Translation | L A A D | R E R | S I H | D E C L | V S K | 15 |
| R35464 | GGTGGTGGGC | AGATTCCGGG | CCTCCATGCC | TAGGTGGTGG | TACAATGTCA | 150 |
| N39798 | GGTGGTGGGC | AGATGCCGGG | CCTCCATGCC | TAGGTGGTGG | TACAATGTCA | 127 |
| H94519 | GGTGGTGGGC | AGATGCCGGG | CCTCCATGCC | TAGGTGGTGG | TACAATGTCA | 146 |
| R74593 corr. | ----- | ----- | ----- | ----- | ----- | |
| Consensus | GGTGGTGGGC | AGATGCCGGG | CCTCCATGCC | TAGGTGGTGG | TACAATGTCA | 149 |
| Translation | Y Y G | R C R A | S M P | R W W | Y N Y T | 32 |
| R35464 | CTGACGGATC | CTGCCAGCTG | TTTGTGTATG | GGGGCTGTGA | CGGAAACAGC | 200 |
| N39798 | CTGACGGATC | CTGCCAGCTG | TTTGTGTATG | GGGGCTGTGA | CGGAAACAGC | 177 |
| H94519 | CTGACGGATC | CTGCCAGCTG | TTTGTGTATG | GGGGCTGTGA | CGGAAACAGC | 196 |
| R74593 corr. | ----- | ----- | ----- | ----- | -----GC | 2 |
| Consensus | CTGACGGATC | CTGCCAGCTG | TTTGTGTATG | GGGGCTGTGA | CGGAAACAGC | 199 |
| Translation | D G S | C Q L | F Y Y G | G C D | G N S | 48 |
| R35464 | AATAATTACC | TGACCAAGGA | GGAGTGCCTC | AAGAAATGTG | CCACTGTCAC | 250 |
| N39798 | AATAATTACC | TGACCAAGGA | GGAGTGCCTC | AAGAAATGTG | CCACTGTCAC | 227 |
| H94519 | AATAATTACC | TGACCAAGGA | GGAGTGCCTC | AAGAAATGTG | CCACTGTCAC | 246 |
| R74593 corr. | AATAATTACC | TGACCAAGGA | GGAGTGCCTC | AAGAAATGTG | CCACTGTCAC | 52 |
| Consensus | AATAATTACC | TGACCAAGGA | GGAGTGCCTC | AAGAAATGTG | CCACTGTCAC | 249 |
| Translation | N N Y L | T K E | E C L | K K C A | T V T | 65 |
| R35464 | AGAGAATGCC | ACGGGTGACC | TGGCCACCAG | CAGGAATGCA | GCGGATTTCCT | 300 |
| N39798 | AGAGAATGCC | ACGGGTGACC | TGGCCACCAG | CAGGAATGCA | GCGGATTTCCT | 277 |
| H94519 | AGAGAATGCC | ACGGGTGACC | TGGCCACCAG | CAGGAATGCA | GCGGATTTCCT | 296 |
| R74593 corr. | AGAGAATGCC | ACGGGTGACC | TGGCCACCAG | CAGGAATGCA | GCGGATTTCCT | 102 |
| Consensus | AGAGAATGCC | ACGGGTGACC | TGGCCACCAG | CAGGAATGCA | GCGGATTTCCT | 299 |
| Translation | E N A | T G D L | A T S | R N A | A D S S | 82 |
| R35464 | CTGTCCCAAG | TGCTCCCAGA | AGGCAGGATT | CTTGAAGACC | ACTTCAGCGA | 350 |
| N39798 | CTGTCCCAAG | TGCTCCCAGA | AGGCAGGATT | CT-GAAGACC | ACTCCAGCGA | 326 |
| H94519 | CTGTCCCAAG | TGCTCCCAGA | AGGCAGGATT | CT-GAAGACC | ACTCCAGCGA | 345 |
| R74593 corr. | CTGTCCCAAG | TGCTCCCAGA | AGGCAGGATT | CT-GAAGACC | ACTCCAGCGA | 151 |
| Consensus | CTGTCCCAAG | TGCTCCCAGA | AGGCAGGATT | CT-GAAGACC | ACTCCAGCGA | 348 |
| Translation | V P S | A P R | R Q D S | E D H | S S D | 98 |
| R35464 | TATGTTTCAA | NTATTGNAAG | AATAATTGCA | CCGNCAACGN | ATT----- | 393 |
| N39798 | TATGTT-CAA | CTA-TG-AAG | AATACT-GCA | CCGCCAACGC | AGTCACTGGG | 372 |
| H94519 | TATGTT-CAA | CTA-TG-AAG | AATACTGGCA | CCGCCAACGC | ATTCACTGGG | 392 |
| R74593 corr. | TATGTT-CAA | CTA-TG-AAG | AATACT-GCA | CCGCCAACGC | AGTCACTGGG | 197 |
| Consensus | TATGTT-CAA | CTA-TG-AAG | AATACT-GCA | CCGCCAACGC | AGTCACTGGG | 394 |
| Translation | M F N | Y E E | Y C T | A N A | V T G | 113 |

FIGURE 3 (CONT)

| | | | | | | |
|--------------|-------------------|------------|------------|------------|------------|-----|
| R35464 | ----- | ----- | ----- | ----- | ----- | |
| N39798 | CCTTGC-GTG | GAATCCTTTC | CCACGCTGGN | AATTTNGACG | TTGAGAAGGA | 421 |
| H94519 | CCT-GC-GTG | -CATCCTT-C | CCACGCTGGT | ACTTT-GNCG | ----- | 427 |
| R74593 corr. | CCTTGCCGTG | -CATCCTT-C | CCACGCTGGT | ACTTT-GACG | TGGAGA-GGA | 243 |
| Consensus | CCTTGCCGTG | -CATCCTT-C | CCACGCTGGT | ACTTT-GACG | TGGAGA-GGA | 440 |
| Translation | P C R A | S F | P R W Y | F D V | E R N | 129 |
| | | | | | | |
| R35464 | ----- | ----- | ----- | ----- | ----- | |
| N39798 | AC----- | ----- | ----- | ----- | ----- | 423 |
| H94519 | ----- | ----- | ----- | ----- | ----- | |
| R74593 corr. | ACTCCTGCAA | TAACTTCATC | TATGGAGGCT | GCCGGGGCAA | TAAGAACAGC | 293 |
| Consensus | ACTCCTGCAA | TAACTTCATC | TATGGAGGCT | GCCGGGGCAA | TAAGAACAGC | 490 |
| Translation | S C N | N F I | Y G G C | R G N | K N S | 145 |
| | | | | | | |
| R35464 | ----- | ----- | ----- | ----- | ----- | |
| N39798 | ----- | ----- | ----- | ----- | ----- | |
| H94519 | ----- | ----- | ----- | ----- | ----- | |
| R74593 corr. | TACCGCTCTG | AGGAGGCCTG | CATGCTCCGC | TGCTTCCGCC | AGCAGGAGAA | 343 |
| Consensus | TACCGCTCTG | AGGAGGCCTG | CATGCTCCGC | TGCTTCCGCC | AGCAGGAGAA | 540 |
| Translation | Y R S E | E A C | M L R | C F R Q | Q E N | 162 |
| | | | | | | |
| R35464 | ----- | ----- | ----- | ----- | ----- | |
| N39798 | ----- | ----- | ----- | ----- | ----- | |
| H94519 | ----- | ----- | ----- | ----- | ----- | |
| R74593 corr. | TCCTCCCCTG | CCCCTTGGCT | CAAAGGTGGT | GGTTCTGGCC | GGGGCTGTTT | 393 |
| Consensus | TCCTCCCCTG | CCCCTTGGCT | CAAAGGTGGT | GGTTCTGGCC | GGGGCTGTTT | 590 |
| Translation | P P L | P L G S | K V V | V L A | G A V S | 179 |
| | | | | | | |
| R35464 | ----- | ----- | ----- | ----- | ----- | |
| N39798 | ----- | ----- | ----- | ----- | ----- | |
| H94519 | ----- | ----- | ----- | ----- | ----- | |
| R74593 corr. | CGTGATGGTG | TTGATCCTTT | TCCTGGGGAG | CNTCCATGGT | CTTACTGATT | 443 |
| Consensus | CGTGATGGTG | TTGATCCTTT | TCCTGGGGAG | CNTCCATGGT | CTTACTGATT | 640 |
| Translation | * W C | * S F | S W G A | S M V | L L I | 195 |
| | | | | | | |
| R35464 | ----- | ----- | ----- | ----- | ----- | |
| N39798 | ----- | ----- | ----- | ----- | ----- | |
| H94519 | ----- | ----- | ----- | ----- | ----- | |
| R74593 corr. | CCGGGTGGCA | AGGAGGAACC | AGGAGCGTGC | CCTGCGGANC | GTCTGGAGCT | 493 |
| Consensus | CCGGGTGGCA | AGGAGGAACC | AGGAGCGTGC | CCTGCGGANC | GTCTGGAGCT | 690 |
| Translation | P G G K | E E P | G A C | P A * R | L E L | 212 |
| | | | | | | |
| R35464 | ----- | ----- | | | | |
| N39798 | ----- | ----- | | | | |
| H94519 | ----- | ----- | | | | |
| R74593 corr. | TCGGAGATGA | CAAGGGNT | | | | 511 |
| Consensus | <u>TCGGAGATGA</u> | CAAGGGNT | | | | 708 |
| Translation | R R * | Q G | | | | 217 |

KEY

R35464 = Nucleic acid sequence of EST R35464 (SEQ ID NO.: 12)
 N39798 = Nucleic acid sequence of EST N39798 (SEQ ID NO.: 17)
 H94519 = Nucleic acid sequence of EST H94519 (SEQ ID NO.: 16)
 R74593 corr. = Corrected version of (SEQ ID NO.: 14) G at b.p. 114
 Consensus = Nucleic acid sequence for human bikunin (SEQ ID NO.: 9)
 Translation = Amino acid Translation of Consensus (SEQ ID NO.: 10)

Figure 4 A.

Schematic depicting the overlap of ESTs bearing homology to the cDNA sequence encoding placental bikunin

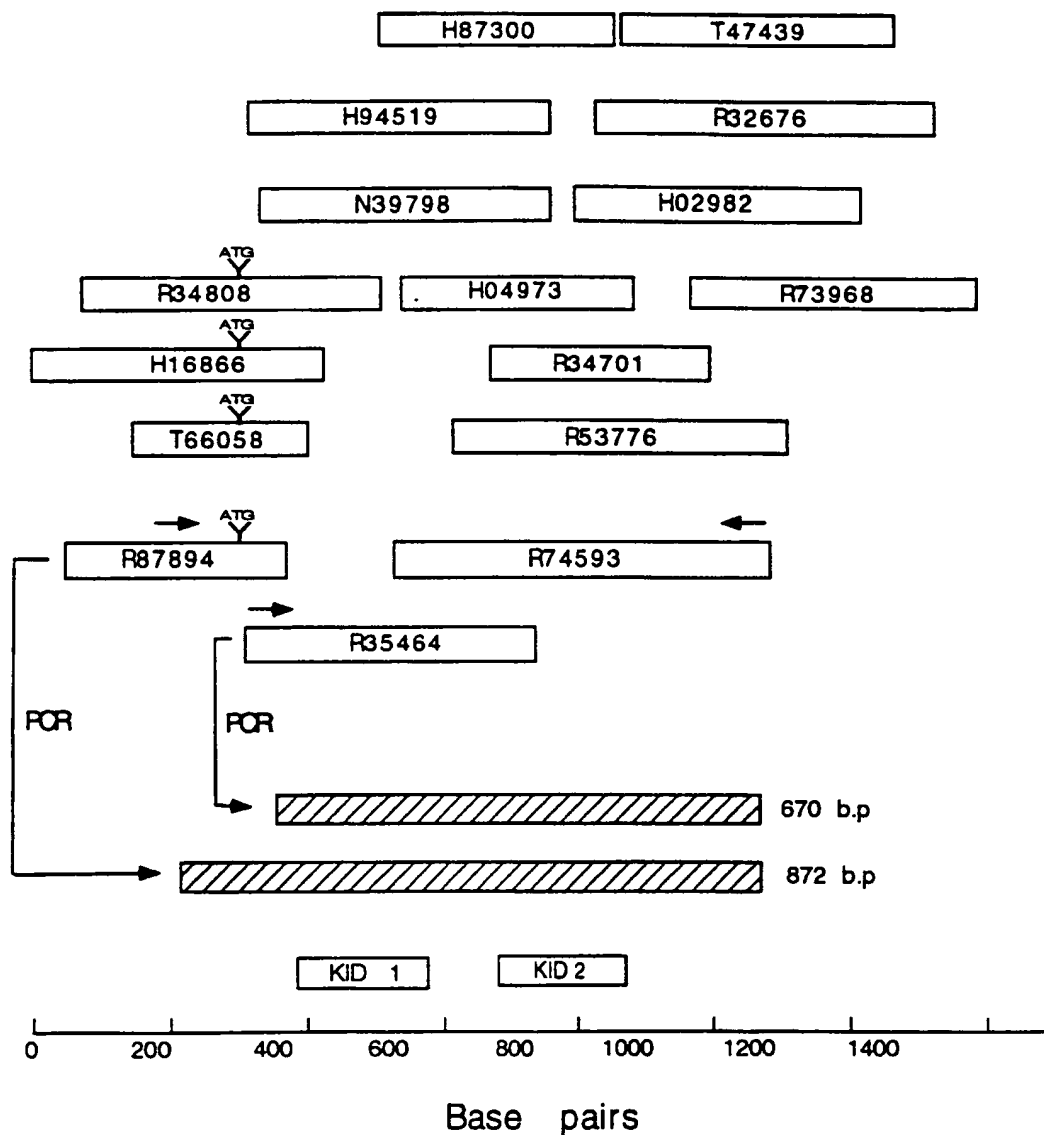


Figure 4B

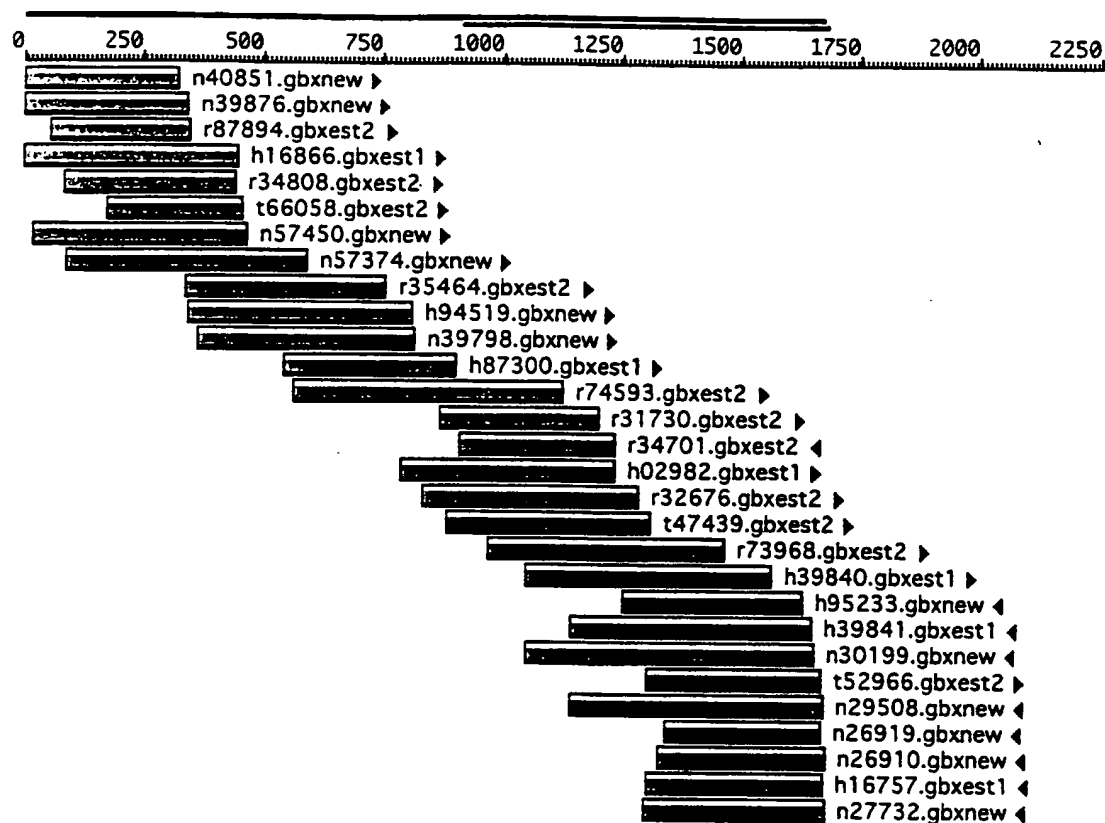


Figure 4C

| | 1 | | | | 50 |
|---------|------------|------------|------------|------------|------------|
| Bikunin |GCGA | CCTCCGCGCG | TTGGGAGGTG | TAGCGCGGCT | CTGAACGCGT |
| N40851 |GCGA | CCTCCGCGCG | TTGGGAGGTG | TAGCGCGGCT | CTGAACGCGT |
| N39876 |GCGA | CCTCCGCGCG | TTGGGAGGTG | TAGCGCGGCT | CTGAACGCGT |
| R87894 | | | | | |
| H16866 |GGCGA | CCTCCGCGCG | TTGGGAGGTG | TAGCGCGGCT | CTGAACGGGN |
| R34808 | | | | | |
| T66058 | | | | | |
| N57450 | | |T | TAGCGCGGCT | CTGAACGCNA |
| N57374 | | | | | |
| R35464 | | | | | |
| H94519 | | | | | |
| N39798 | | | | | |
| H87300 | | | | | |
| R74593 | | | | | |
| R31730 | | | | | |
| R34701 | | | | | |
| H02982 | | | | | |
| R32676 | | | | | |
| T47439 | | | | | |
| R73968 | | | | | |
| H39840 | | | | | |
| H95233 | | | | | |
| H39841 | | | | | |
| N30199 | | | | | |
| T52966 | | | | | |
| N29508 | | | | | |
| N26919 | | | | | |
| N26910 | | | | | |
| H16757 | | | | | |
| N27732 | | | | | |

Figure 4C (Con't)

| | 51 | 100 |
|---------|--|-----|
| Bikunin | GNA GGGCCG TTGAGTGTCTG CAGGCGGCCGA GGGCGCGAGT GAGGAGCAGA | |
| N40851 | NGAGNGGCCG TTGAGTGTCTG CAGGCGGCCGA GGGCGCGAGT GAGGAGCAGA | |
| N39876 | GCA.GGGCCG TTGAGTGTCTG CAGGCGGCCGA GGGCGCGAGT GAGGAGCAGA | |
| R87894 | TTGAGTGTNG NAGGCGGCCGA GGGCGCGAGT GAGGAGCAGA | |
| H16866 | ..ANGGGCCG TTGAGTGTCTG CAGGCGGCC.A GGGCN.CAGT GAGGAGCAGA | |
| R34808 | G GAGGAGCAGA | |
| T66058 | | |
| N57450 | GAAGNGGCCG TTGAGTGTCTG CAGGCGGCCGA GGGCGCGAGT GAGGAGCAGA | |
| N57374 | AGA | |
| R35464 | | |
| H94519 | | |
| N39798 | | |
| H87300 | | |
| R74593 | | |
| R31730 | | |
| R34701 | | |
| H02982 | | |
| R32676 | | |
| T47439 | | |
| R73968 | | |
| H39840 | | |
| H95233 | | |
| H39841 | | |
| N30199 | | |
| T52966 | | |
| N29508 | | |
| N26919 | | |
| N26910 | | |
| H16757 | | |
| N27732 | | |

Figure 4C (Con't)

| | 101 | 150 |
|---------|------------|---|
| Bikunin | CCCAGGCATC | GCGCGCCGAG AAGNC GGGC GTCCCCACAC TGAAGGTCCG |
| N40851 | CCCAGGCATC | GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG |
| N39876 | CCCAGGCATC | GCGCGCCGAG AAGNC.GGGC NTCCCCACAC TGAAGGTCCG |
| R87894 | CCCAGGCATC | GCGCGCCGAG AAGGCCGGGC GTCCCCACAC TGAAGGTCCG |
| H16866 | CCCAGGCATC | GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG |
| R34808 | CCCAGGCATC | GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG |
| T66058 | | |
| N57450 | CCCAGGCATC | GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG |
| N57374 | CCCAGGCATC | GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG |
| R35464 | | |
| H94519 | | |
| N39798 | | |
| H87300 | | |
| R74593 | | |
| R31730 | | |
| R34701 | | |
| H02982 | | |
| R32676 | | |
| T47439 | | |
| R73968 | | |
| H39840 | | |
| H95233 | | |
| H39841 | | |
| N30199 | | |
| T52966 | | |
| N29508 | | |
| N26919 | | |
| N26910 | | |
| H16757 | | |
| N27732 | | |

10/41

Figure 4C (Con't)

| | 151 | | | | 200 |
|---------|------------|------------|------------|------------|------------|
| Bikunin | GAAAGGCGAC | TTCCGGGGGC | TTTGGCACCT | GGCGGACCCT | CCCGGAGCGT |
| N40851 | GAAAGGCGAC | TTCCGGGGGC | TTTGGCACCT | GGCGGACCCT | CCCGGAGCGT |
| N39876 | GAAAGGCGAC | TTCCGGGGGC | TTTGGCACCT | GGCGGACCCT | CCCGGAGCGT |
| R87894 | GAAAGGCGAC | TTCCGGGGGC | TTTGGCACCT | GGCGGACCCT | CCCGGAGCGT |
| H16866 | GAAAGGCGAC | TTCCGGGGGC | TTTGGCACCT | GGCGGACG.T | CCCGGAGCN. |
| R34808 | GAAAGGCGAC | TTCCGGGGGC | TTTGGCACCT | GGCGGACCCT | CCCGGAGCGT |
| T66058 | | | | ...GGACCCT | CCCGGAGCGT |
| N57450 | GAAAGGCGAC | TTCCGGGGGC | TTTGGCACCT | GGCGGACCCT | CCCGGAGCGT |
| N57374 | GAAAGGCGAC | TTCCGGGGGC | TTTGGCACCT | GGCGGACCCT | CCCGGAGCGT |
| R35464 | | | | | |
| H94519 | | | | | |
| N39798 | | | | | |
| H87300 | | | | | |
| R74593 | | | | | |
| R31730 | | | | | |
| R34701 | | | | | |
| H02982 | | | | | |
| R32676 | | | | | |
| T47439 | | | | | |
| R73968 | | | | | |
| H39840 | | | | | |
| H95233 | | | | | |
| H39841 | | | | | |
| N30199 | | | | | |
| T52966 | | | | | |
| N29508 | | | | | |
| N26919 | | | | | |
| N26910 | | | | | |
| H16757 | | | | | |
| N27732 | | | | | |

Figure 4C (Con't)

| | 201 | | | | 250 |
|---------|------------|------------|------------|------------|------------|
| Bikunin | CGGCACCTGA | ACGCGAGGCG | CTCCATTGCG | CGTGCGTTTG | .AGGGGCTTC |
| N40851 | CGGCACCTGA | ACGCGAGGCG | CTCCATTGCG | CGTGCGTNTG | .AGGGGCTTC |
| N39876 | CGGCACCTGA | ACGCGAGGCG | CTCCATTGCG | CGTGCGTTTG | .AGGGGCTTC |
| R87894 | CGGCACCTGA | ACGCGAGGCG | CTCCATTGCG | CGTGCGTTTG | .AGGGGCTTC |
| H16866 | .GGCACCTGA | ACGCGAGGCG | CTCCATTGCG | CGTGCGTTTG | .AGGGGCTTC |
| R34808 | CGGCACCTGA | ACGCGAGGCG | CTCCATTGCG | CGTGCGTNTG | GAGGGGCTTC |
| T66058 | CGGCACCTGA | ACGCGAGGCG | CTCCATTGCG | .GTGCGTGTG | NAGGGGCTTC |
| N57450 | CGGCACCTGA | ACGCGAGGCG | CTCCATTGCG | CGTGCGTTTG | .AGGGGCTTC |
| N57374 | CGGCACCTGA | ACGCGAGGCG | CTCCATTGCG | CGTGCGTTTG | .AGGGGCTTC |
| R35464 | | | | | |
| H94519 | | | | | |
| N39798 | | | | | |
| H87300 | | | | | |
| R74593 | | | | | |
| R31730 | | | | | |
| R34701 | | | | | |
| H02982 | | | | | |
| R32676 | | | | | |
| T47439 | | | | | |
| R73968 | | | | | |
| H39840 | | | | | |
| H95233 | | | | | |
| H39841 | | | | | |
| N30199 | | | | | |
| T52966 | | | | | |
| N29508 | | | | | |
| N26919 | | | | | |
| N26910 | | | | | |
| H16757 | | | | | |
| N27732 | | | | | |

Figure 4C (Con't)

| | 251 | 300 |
|---------|--|-----|
| Bikunin | CCGCACCT G ATCGCGAGAC CCCAACGGCT GGTGG CGTC GC TG CGCG | |
| N40851 | CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTGG.CGTC GCCTG.CGCG | |
| N39876 | CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTGG.CGTC GCCTG.CGCG | |
| R87894 | CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTNG.CGTC GC.TN.CGCG | |
| H16866 | CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTNG.CGTC GC.TGGCGCG | |
| R34808 | CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTGGGCGTC GC.TG.CGCG | |
| T66058 | CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTGG.CGTC GC.TG.CGCG | |
| N57450 | CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTGG.CGTC GCCTG.CGCG | |
| N57374 | CCGGAACTTG ATCGCGAGAC CCCAACGGCT GGTGG.CGTC GC.TG.CGCG | |
| R35464 | | |
| H94519 | | |
| N39798 | | |
| H87300 | | |
| R74593 | | |
| R31730 | | |
| R34701 | | |
| H02982 | | |
| R32676 | | |
| T47439 | | |
| R73968 | | |
| H39840 | | |
| H95233 | | |
| H39841 | | |
| N30199 | | |
| T52966 | | |
| N29508 | | |
| N26919 | | |
| N26910 | | |
| H16757 | | |
| N27732 | | |

Figure 4C (Con't)

| | 301 | | | | 350 |
|---------|------------|------------|------------|------------|------------|
| Bikunin | TC TCGGCTG | AGCT GGCCA | TGGCGCANT | GTTGC GGGC | T GAGGC GG |
| N40851 | TC.TCGGCTG | AGCT.GGNCA | TGTCG | | |
| N39876 | TC.TCGGCTG | AGCT.GGCCA | TGGCGCACT. | G.TGCGGNGC | T.GAGGC.G |
| R87894 | TC.TCGGCTG | AGCTTGGCCA | TGGCGCANT. | GTTNC.GGGC | T.NAGGC.GG |
| H16866 | TTCTCGGCTG | AGCT.GGCCA | TGGCGCANT. | GTTGC.GNGC | T.GAGGC.GG |
| R34808 | TCTTCGGCTG | AGCTGGGCCA | TGGCGCANTT | GTTGC.GGGC | T.GAGGC.GG |
| T66058 | TC.TCGGCTG | AGCT.GGCCA | TGGCGCANT. | GTTGC.GNGC | T.GAGGC.GG |
| N57450 | TC.TCGGCTG | AGCT.GGCCA | TGGCGCANT. | GGTGC.GGGC | TTGAGGC.GG |
| N57374 | TCCTCGGCTG | AGCT.GGCCA | TGGCGCANT. | GGTGCCGNGC | T.GAGGCCGG |
| R35464 | | | | |GGCCGG |
| H94519 | | | | | |
| N39798 | | | | | |
| H87300 | | | | | |
| R74593 | | | | | |
| R31730 | | | | | |
| R34701 | | | | | |
| H02982 | | | | | |
| R32676 | | | | | |
| T47439 | | | | | |
| R73968 | | | | | |
| H39840 | | | | | |
| H95233 | | | | | |
| H39841 | | | | | |
| N30199 | | | | | |
| T52966 | | | | | |
| N29508 | | | | | |
| N26919 | | | | | |
| N26910 | | | | | |
| H16757 | | | | | |
| N27732 | | | | | |

Figure 4C (Con't)

| | 351 | | | 400 |
|---------|------------|------------|----------------------------------|-----|
| Bikunin | AC GG CG | TTTCTCG | CC TGCTGGG A TCGCT GC T CCTCTCT | |
| R87894 | ACG. | | | |
| H16866 | AC..CGNCGT | TTTTCTTCG. | CCTTGCTGGG ATTGCTTGC TTCCTNTCTG | |
| R34808 | ACGCCGNCG. | TTTTTTCGN | CCTTGCTGGG ATTGCTTG. TTNCTCTCTN | |
| T66058 | ...CGGNCG. | TTTTCTCG. | CC.TGCTGGG A.TCGCT.GC T.CCTCTCT. | |
| N57450 | ANN.NGCCG. | ..TTTCTCG. | CC.TGCTGGG A.TCGCT.GC T.CCTCTCT. | |
| N57374 | AG..GGCCCG | ..TTTCTCG. | CCTTGCTGGG A.TCGCT.GC T.CCTCTCTG | |
| R35464 |GTCG. | ..TTTCTCG. | CCTGGCTGGG A.TCGCT.GC T.CCTCTCT. | |
| H94519 | .GCNGCGCG. | ..TTNNTCG. | CN.TGCTGGG A.TCGCT.GC A.CCTCTCT. | |
| N39798 | |CTGGG | ANTCGCT.GC T.CCTCTCT. | |
| H87300 | | | | |
| R74593 | | | | |
| R31730 | | | | |
| R34701 | | | | |
| H02982 | | | | |
| R32676 | | | | |
| T47439 | | | | |
| R73968 | | | | |
| H39840 | | | | |
| H95233 | | | | |
| H39841 | | | | |
| N30199 | | | | |
| T52966 | | | | |
| N29508 | | | | |
| N26919 | | | | |
| N26910 | | | | |
| H16757 | | | | |
| N27732 | | | | |

Figure 4C (Con't)

| | 401 | 450 |
|---------|--|-----|
| Bikunin | GGGG TCCTG G CGGCCGA CCGA GAACG CA GCA TCC ACGACTT CT | |
| H16866 | GGGGTTCCTG GG.CGGCCGA CCGA.GAACG CA.GCA.TCC AAGAATTTT | |
| R34808 | GGGGTTC.TG GGGNGGCCGA NCGA.GAACG CAAGCA.TTC ACGA.TTT | |
| T66058 | GGGG.TCCTG G..CGGCCGA CCGA.GAACG CA.GCA.TCC ACGANTT.CT | |
| N57450 | GGGG.TCCTG G..CGGCCGA CCGA.GAACG CA.GCA.TCC ACGACTT.CT | |
| N57374 | GGGG.TCCTG G..CGGCCGA NCGAAGAANG CA.GCAATCC ANGAATTNCT | |
| R35464 | GGGG.TCCTG G.CCGGCCGA CCGA.GAACG CA.GCA.TCC ACGACTT.CT | |
| H94519 | GGGG.TCGNG G..CGGCCGA CCGA.GAACG CA.GCA.TCC ACGACTT.CT | |
| N39798 | GGGG.TCCTG G..CGGCCGA CCGA.GAACG CA.GCA.TCC ACGACTT.CT | |
| H87300 | | |
| R74593 | | |
| R31730 | | |
| R34701 | | |
| H02982 | | |
| R32676 | | |
| T47439 | | |
| R73968 | | |
| H39840 | | |
| H95233 | | |
| H39841 | | |
| N30199 | | |
| T52966 | | |
| N29508 | | |
| N26919 | | |
| N26910 | | |
| H16757 | | |
| N27732 | | |

Figure 4C (Con't)

| | 451 | | 500 |
|---------|-----------|----------------------------------|------------|
| Bikunin | GCCTGGTGT | CGAAGGT GG TGGGCAGATG CCGGG CCTC | CATGCCTA G |
| H16866 | GCC | | |
| T66058 | TCCTGGTGT | CGAAGG | |
| N57450 | GCCTGGTGT | CGAAGGT.GG TGGGCAG | |
| N57374 | GCCTGGTGT | CGAAAGTTGG TGGGCANATT CCGGGGCCTT | CATGNCTAAG |
| R35464 | GCCTGGTGT | CGAAGGT.GG TGGGCAGATT CCGGG.CCTC | CATGCCTA.G |
| H94519 | GCCTGGTGT | CGAAGGT.GG TGGGCAGATG CCGGG.CCTC | CATGCCTA.G |
| N39798 | GCCTGGTGT | CGAAGGT.GG TGGGCAGATG CCGGG.CCTC | CATGCCTA.G |
| H87300 | | | |
| R74593 | | | |
| R31730 | | | |
| R34701 | | | |
| H02982 | | | |
| R32676 | | | |
| T47439 | | | |
| R73968 | | | |
| H39840 | | | |
| H95233 | | | |
| H39841 | | | |
| N30199 | | | |
| T52966 | | | |
| N29508 | | | |
| N26919 | | | |
| N26910 | | | |
| H16757 | | | |
| N27732 | | | |

17/41

Figure 4C (Con't)

| | | | | | |
|---------|-------------|------------|------------|------------|------------|
| | 501 | | | | 550 |
| Bikunin | G TGGT GGT | ACAATGTCAC | TGACGGATCC | TGCCAGCTGT | TTGTGT ATG |
| N57374 | GTTGGTITGGT | ANAATGTNAA | TTAANGATTG | TTGCAACTGT | TTGTGTNATT |
| R35464 | G.TGGT.GGT | ACAATGTCAC | TGACGGATCC | TGCCAGCTGT | TTGTGT.ATG |
| H94519 | G.TGGT.GGT | ACAATGTCAC | TGACGGATCC | TGCCAGCTGT | TTGTGT.ATG |
| N39798 | G.TGGT.GGT | ACAATGTCAC | TGACGGATCC | TGCCAGCTGT | TTGTGT.ATG |
| H87300 | | | | | |
| R74593 | | | | | |
| R31730 | | | | | |
| R34701 | | | | | |
| H02982 | | | | | |
| R32676 | | | | | |
| T47439 | | | | | |
| R73968 | | | | | |
| H39840 | | | | | |
| H95233 | | | | | |
| H39841 | | | | | |
| N30199 | | | | | |
| T52966 | | | | | |
| N29508 | | | | | |
| N26919 | | | | | |
| N26910 | | | | | |
| H16757 | | | | | |
| N27732 | | | | | |
| | 551 | | | | 600 |
| Bikunin | GGGGCTGTGA | CGGAAACA | GCAATAATTA | CCTGACCAAG | GA GGAGTGC |
| N57374 | GGGGCTNTTA | AACGGAAANA | .CAATAATNA | CCTGACCAAA | GAAGNAAT.. |
| R35464 | GGGGCTGTGA | ..CGGAAACA | GCAATAATTA | CCTGACCAAG | GA.GGAGTGC |
| H94519 | GGGGCTGTGA | ..CGGAAACA | GCAATAATTA | CCTGACCAAG | GA.GGAGTGC |
| N39798 | GGGGCTGTGA | ..CGGAAACA | GCAATAATTA | CCTGACCAAG | GA.GGAGTGC |
| H87300 | GATTCGGCAC | AGGGGAAACA | GCAATAATTA | CCTGACCAAG | GA.GGAGTNC |
| R74593 | | | GCAATAATTA | CCTGACCAAG | GA.GGAGTGC |
| R31730 | | | | | |
| R34701 | | | | | |
| H02982 | | | | | |
| R32676 | | | | | |
| T47439 | | | | | |
| R73968 | | | | | |
| H39840 | | | | | |
| H95233 | | | | | |
| H39841 | | | | | |
| N30199 | | | | | |
| T52966 | | | | | |
| N29508 | | | | | |
| N26919 | | | | | |
| N26910 | | | | | |
| H16757 | | | | | |
| N27732 | | | | | |

18/41

Figure 4C (Con't)

| | | | | | |
|---------|------------|------------|------------|------------|------------|
| | 601 | | | | 650 |
| Bikunin | CTCAAGAAAT | GTGCCACTGT | CACAGAGAAT | GCCACGGGTG | ACCTGGCCAC |
| R35464 | CTCAAGAAAT | GTGCCACTGT | CACAGAGAAT | GCCACGGGTG | ACCTGGCCAC |
| H94519 | CTCAAGAAAT | GTGCCACTGT | CACAGAGAAT | GCCACGGGTG | ACCTGGCCAC |
| N39798 | CTCAAGAAAT | GTGCCACTGT | CACAGAGAAT | GCCACGGGTG | ACCTGGCCAC |
| H87300 | CTCAAGAAAT | GTNCCACTGT | CACAGAGAAT | GCCACGGGTG | ACCTGGCCAC |
| R74593 | CTCAAGAAAT | GTGCCACTGT | CACAGAGAAT | GCCACGGGTG | ACCTGGCCAC |
| R31730 | | | | | |
| R34701 | | | | | |
| H02982 | | | | | |
| R32676 | | | | | |
| T47439 | | | | | |
| R73968 | | | | | |
| H39840 | | | | | |
| H95233 | | | | | |
| H39841 | | | | | |
| N30199 | | | | | |
| T52966 | | | | | |
| N29508 | | | | | |
| N26919 | | | | | |
| N26910 | | | | | |
| H16757 | | | | | |
| N27732 | | | | | |
| | 651 | | | | 700 |
| Bikunin | CAGCAGGAAT | GCAGCGGATT | CCTCTGTCCC | AAGTGCTCCC | AGAAGGCAGG |
| R35464 | CAGCAGGAAT | GCAGCGGATT | CCTCTGTCCC | AAGTGCTCCC | AGAAGGCAGG |
| H94519 | CAGCAGGAAT | GCAGCGGATT | CCTCTGTCCC | AAGTGCTCCC | AGAAGGCAGG |
| N39798 | CAGCAGGAAT | GCAGCGGATT | CCTCTGTCCC | AAGTGCTCCC | AGAAGGCAGG |
| H87300 | CAGCAGGAAT | GCAGCGGATT | CCTCTGTCCC | AAGTGCTCCC | AGAAGGCAGG |
| R74593 | CAGCAGGAAT | GCAGCGGATT | CCTCTGTCCC | AAGTGCTCCC | AGAAGGCAGG |
| R31730 | | | | | |
| R34701 | | | | | |
| H02982 | | | | | |
| R32676 | | | | | |
| T47439 | | | | | |
| R73968 | | | | | |
| H39840 | | | | | |
| H95233 | | | | | |
| H39841 | | | | | |
| N30199 | | | | | |
| T52966 | | | | | |
| N29508 | | | | | |
| N26919 | | | | | |
| N26910 | | | | | |
| H16757 | | | | | |
| N27732 | | | | | |

19/41

Figure 4C (Con't)

| | | | |
|---------|--|--|-----|
| | 701 | | 750 |
| Bikunin | ATTCT GAAG ACCACTCCAG CGATATGTT CAACTAT G AAGAATACTG | | |
| R35464 | ATTCTTGAAG ACCACTTCAG CGATATGTTT CAANTATTGN AAGAATAATT | | |
| H94519 | ATTCT.GAAG ACCACTCCAG CGATATGTT. CAACTAT..G AAGAATACTG | | |
| N39798 | ATTCT.GAAG ACCACTCCAG CGATATGTT. CAACTAT..G AAGAATACTG | | |
| H87300 | ATTCT.GAAG ACCACTCCAG CGATATGTT. CAACTAT..G AAGAATACTG | | |
| R74593 | ATTCT.GAAG ACCACTCCAG CGATATGTT. CAACTAT..G AAGAATACTG | | |
| R31730 | | | |
| R34701 | | | |
| H02982 | | | |
| R32676 | | | |
| T47439 | | | |
| R73968 | | | |
| H39840 | | | |
| H95233 | | | |
| H39841 | | | |
| N30199 | | | |
| T52966 | | | |
| N29508 | | | |
| N26919 | | | |
| N26910 | | | |
| H16757 | | | |
| N27732 | | | |
| | 751 | | 800 |
| Bikunin | CACCGCCAA CGCAGT CAC TGGGCC TTG CCGTG CAT CCTT CCCAC | | |
| R35464 | GCACCGNCAA CGNATT | | |
| H94519 | GCACCGCCAA CGCATT.CAC TGGGCC..TG C.GTG.CAT. CCTT.CCCAC | | |
| N39798 | .CACCGCCAA CGCAGT.CAC TGGGGCCTTG C.GTGGAAAT. CCTTTCCAC | | |
| H87300 | .CACCGCCAA CGCAGTNCAC TGGGCC.TTG C.GTGGCATN CCTT.CCCAC | | |
| R74593 | .CACCGCCAA CGCAGT.CAC TGGGCC.TTG CCGTG.CAT. CCTT.CCCAC | | |
| R31730 | | | |
| R34701 | | | |
| H02982 | | | |
| R32676 | | | |
| T47439 | | | |
| R73968 | | | |
| H39840 | | | |
| H95233 | | | |
| H39841 | | | |
| N30199 | | | |
| T52966 | | | |
| N29508 | | | |
| N26919 | | | |
| N26910 | | | |
| H16757 | | | |
| N27732 | | | |

20/41

Figure 4C (Con't)

| | | | |
|---------|--|--|-----|
| | 801 | | 850 |
| Bikunin | GCTGGTACTT T GACGTGGA GA GGAATC CTG CAATAA CTTTCATCTAT | | |
| H94519 | GCTGGTACTT T.GNCGT | | |
| N39798 | GCTGGNAATT TNGACGTGA GAAGGAAC | | |
| H87300 | GCTNGTACTT T.GACGTGGA GA.GGAATC CTGGCAATAA CTTTCATCTAT | | |
| R74593 | GCTGGTACTT T.GACGTGGA GA.GGAATC CTG.CAATAA CTTTCATCTAT | | |
| R31730 | | | |
| R34701 | | | |
| H02982 |GA GA.GGAATC CTG.CAATAA CTTTCATCTAT | | |
| R32676 |G ATTC..GGAA | | |
| T47439 | | | |
| R73968 | | | |
| H39840 | | | |
| H95233 | | | |
| H39841 | | | |
| N30199 | | | |
| T52966 | | | |
| N29508 | | | |
| N26919 | | | |
| N26910 | | | |
| H16757 | | | |
| N27732 | | | |
| | 851 | | 900 |
| Bikunin | GGAGGCT GC CGGGGCAAT AAGAACAG C TACCGCTC T GAGGAGGCCT | | |
| H87300 | GGAGGCTTGC CGGGGCAATN AAGAACAGNT TACCGCTCTT TAGGAGGCCT | | |
| R74593 | GGAGGCT.GC CGGGGCAAT. AAGAACAG.C TACCGCTC.T GAGGAGGCCT | | |
| R31730 |G.C TACCGCTC.T GAGGAGGCCT | | |
| R34701 | | | |
| H02982 | GGNGGCT.GC CGGGG.AAT. AAGAACA.NC TACCGCTC.T GAGGAGGCCT | | |
| R32676 | CGAGGA..GC CGGGGCAAT. AAGAACAG.C TACCGCTC.T GAGGAGGCCT | | |
| T47439 |NGGCCT | | |
| R73968 | | | |
| H39840 | | | |
| H95233 | | | |
| H39841 | | | |
| N30199 | | | |
| T52966 | | | |
| N29508 | | | |
| N26919 | | | |
| N26910 | | | |
| H16757 | | | |
| N27732 | | | |

21/41

Figure 4C (Con't)

| | | | |
|---------|---|--|------------|
| | 901 | | 950 |
| Bikunin | GCA TGCTC CGCTGCTTCC GC | | CA GCAGGA |
| H87300 | .GCA.T.... | | |
| R74593 | .GCA.TGCTC CGCTGCTTCC GC..... | | .CA.GCAGGA |
| R31730 | .GCA.TGCTC CGCTGCTTCC GC..... | | .CA.GCAGGA |
| R34701 |TTCC GC..... | | .CAAGCAGGA |
| H02982 | .GCG.TGCTC CGCTGCTTCC GCTGTGTGT CTCTTCCAGG | | CCA.GCAGGA |
| R32676 | .GCA.TGCTC CGCTGCTTCC GC..... | | .CA.GCAGGA |
| T47439 | TGCAGTGCTC CGCTGCTTCC GC..... | | .CA.GCAGGA |
| R73968 | | | |
| H39840 | | | |
| H95233 | | | |
| H39841 | | | |
| N30199 | | | |
| T52966 | | | |
| N29508 | | | |
| N26919 | | | |
| N26910 | | | |
| H16757 | | | |
| N27732 | | | |
| | 951 | | 1000 |
| Bikunin | GAA TCCTCC CCTGCCCCCTT GGCTCAAAGG TGGTGGTTC | | TGG CGGGGC |
| R74593 | GAA.TCCTCC CCTGCCCCCTT GGCTCAAAGG TGGTGGTTC | | TGGCGGGGC |
| R31730 | GAA.TCCTCC CCTGCCCCCTT GGCTCAAAGG TGGTGGTTC | | TGG.CGGGGC |
| R34701 | AAANTCCTCC CCTCCCCCTT GGCTCAAAGG TGGTGGTTC | | TGG.CGGGGC |
| H02982 | GAA.TCCTCC CCTGCCCCCTT GGCTCAAAGG TGGTGGTTC | | TGG.CGGGGC |
| R32676 | GAA.TCCTCC CCTGCCCCCTT GGCTCAAAGG TGGTGGTTC | | TGG.CGGGGC |
| T47439 | GAA.TCCTCC CCTGCCCCCTT GGCTCAAAGG TGGTGGTTC | | TGG.CGGGGC |
| R73968 | | |CGGGGC |
| H39840 | | | |
| H95233 | | | |
| H39841 | | | |
| N30199 | | | |
| T52966 | | | |
| N29508 | | | |
| N26919 | | | |
| N26910 | | | |
| H16757 | | | |
| N27732 | | | |

22/41

Figure 4C (Con't)

| | 1001 | | 1050 |
|---------|---|--|------|
| Bikunin | TGTT CGTGA TGGTGTGAT CC T CTCC TGGG AGCCT CC ATGGTC | | |
| R74593 | TGTTTCGTGA TGGTGTGAT CCTT..TTCC TGGGGAGCCT CC.ATGGTCT | | |
| R31730 | TGTT.CGTGA TGGTGTGAT CC.T.CTCC TGGGGAGCCT CC.ATGGTC. | | |
| R34701 | TGTT.CGTGA TGGTGTGAT CCTCCTTCC CGGG.AGCCT CCCATGGTCC | | |
| H02982 | TGTT.CGTGA TGGTGTGAT CC.T.CTCC TGGG.AGCCT CC.ATGGTN. | | |
| R32676 | TGTT.CGTGA TGGTGTGAT CC.T.CTCC TGGG.AGCCT CC.ATGGTC. | | |
| T47439 | TGTT.CGTGA TGGTGTGAT CC.T.CTCC TGGG.AGCCT CC.ATGGTC. | | |
| R73968 | TGTT.CGTGA TGGTGTGAT CC.T.CTCC TGGG.AGCCT CC.ATGGTC. | | |
| H39840 | | | |
| H95233 | | | |
| H39841 | | | |
| N30199 | | | |
| T52966 | | | |
| N29508 | | | |
| N26919 | | | |
| N26910 | | | |
| H16757 | | | |
| N27732 | | | |

| | 1051 | | 1100 |
|---------|--|--|------|
| Bikunin | TACC TGAT CCGGGTGGCA CGGAGG AAC C AGG AGCG TGCCCTGCGC | | |
| R74593 | TAC..TGATT CCGGGTGGCA AGGAGG.AAC C.AGG.AGCG TGCCCTGCGG | | |
| R31730 | TACC.TGAT. CCGGGTGGCA CGGAGGGAAC C.AGGAGCG TGCCCTGCGC | | |
| R34701 | TACCCTGAT. CCGGGTGGCA CGGAGG.AAC CCAGG.ANCG TGCCCTGCGC | | |
| H02982 | TACC.TGAT. CCGGGTNGCA CGGAGG.AAC C.AGGAGCG TGCCCTGCGN | | |
| R32676 | TACC.TGAT. CCGGGTGGCA CGGAGG.AAC C.AGGAGCG TGCCCTGCGC | | |
| T47439 | TACC.TGAT. CCGGGTNGCA CGGAGG.AAC C.AGG.AGCG TGCCCTGCGC | | |
| R73968 | TACC.TGAT. CCGGGTGGCA CGGAGG.AAC C.AGG.AGCG TGCCCTGCGC | | |
| H39840 | | | |
| H95233 | | | |
| H39841 | | | |
| N30199 | | | |
| T52966 | | | |
| N29508 | | | |
| N26919 | | | |
| N26910 | | | |
| H16757 | | | |
| N27732 | | | |

23/41

Figure 4C (Con't)

| | | | |
|---------|---|--|------|
| | 1101 | | 1150 |
| Bikunin | ACCG TCT G GAGCTCCGGA GATGACAAGG AGCAGCTGG TGAAGAAC | | |
| R74593 | ANCG.TCT.G GAGCTTCGGA GATGACAAGG GNT | | |
| R31730 | ACCG.TCTGG GAGCTCCGGA GATGACAAGG GAGCAGCTGG GTGAAGAAC. | | |
| R34701 | ACCG.TCT.G GAGCTCCGGA GATGACAAGG .AGCAGCTGG .TGAAGAAC. | | |
| H02982 | ACCG.TCTNG GAGCTCCGGA GATGACAAGG .AGCAGCTGG .TGAAGAAC. | | |
| R32676 | ACCG.TCTGG GAGCTCCGGA GATGACAAGG GAGCAGCTGG .TGAAGAAC. | | |
| T47439 | ACCG.TCT.G GAGCTCCGGA GATGACAAGG .AGCAGCTGG .TGAAGAAC. | | |
| R73968 | ACCG.TCT.G GAGCTCCGGA GATGACAAGG .AGCAGCTGG .TGAAGAAC. | | |
| H39840 | ACCGGTCT.G GAGCTCCGGA GATGACAAGG .AGCAGCTGG .TGAAGAAC. | | |
| H95233 | | | |
| H39841 | | | |
| N30199 | ACCG.TCT.G GAGCTCCGGA GATNACAANG .AGCAGCTGN .TGAAGAACC | | |
| T52966 | | | |
| N29508 | | | |
| N26919 | | | |
| N26910 | | | |
| H16757 | | | |
| N27732 | | | |
| | 1151 | | 1200 |
| Bikunin | ACATATGT C CTGT GACCG CCCTGT CGC C AAGAGG A CT GGGGAA | | |
| R31730 | ACATATGTTT CTGTTGACCG NCCTGTTTCGC C.AAGAGG.A TTGGGGGAA. | | |
| R34701 | ACATATGT.C CTGT.GACCG CCCTGT.CGC C.AAGAGG.A CT.GGGGAA. | | |
| H02982 | ACATATGT.C CTGT.GACCG NCCTGTTTCGN C.AAGAGG.A CTNGGGGAAA | | |
| R32676 | ACATATGTTT CTGTTGACCG CCCTGTTTCGC C.AAGAGGGA NTGGGGGAA. | | |
| T47439 | ACATATGT.C CTGT.GACCG CCCTGT.CGC C.AAGAGG.A CT.GGGGAA. | | |
| R73968 | ACATATGT.C CTGT.GACCG CCCTGT.CGC C.AAGAGG.A CT.GGGGAA. | | |
| H39840 | ACATATGT.C CTGT.GACCG CCCTGT.CGC C.AAGAGG.A CT.NGGGAA. | | |
| H95233 | | | |
| H39841 |C. CCCTGT.CGC CCAAAGG.A CT.GGGGAA. | | |
| N30199 | ACATATGT.C CTGT.GACCG CCCTNT.CGC C.AAGAGG.A CT.GGGNAAA | | |
| T52966 | | | |
| N29508 |CC. CCCTNT.CGC C.AAGAGG.A CT.GGG.AA. | | |
| N26919 | | | |
| N26910 | | | |
| H16757 | | | |
| N27732 | | | |

24/41

Figure 4C (Con't)

| | | | |
|---------|------------|------------|----------------------------------|
| | 1201 | | 1250 |
| Bikunin | GGGAGGGG | AGACTAT G | TGT GA GCT TTTTTT AA A TAGA GG |
| R31730 | .GGGAGGGG | A | |
| R34701 | .GGGAGGGG. | AGACTAT.G. | TGT.GA.GCT TTTTTT..AA A.TA |
| H02982 | GGGAGGGG. | AGATTAT.G. | TGTTGA.GTT TTTTTT..AA ANTAG |
| R32676 | GGGAGGGGG | AGANTATTGT | TGTTGA.GNT TTTTTTAAA ATTAGGAGGG |
| T47439 | .GGGAGGGG. | AGACTAT.G. | TGT.GA.GCT TTTTTT..AA A.TAGA..GG |
| R73968 | .GGGAGGGG. | AGACTAT.G. | TGT.GA.GCT TTTTTT..AA A.TAGA..GG |
| H39840 | .GGGAGGGG. | AGACTAT.G. | TGT.GA.GCT TTTTTT..AA A.TAGA..GG |
| H95233 | | | |
| H39841 | .GGGAGGGGA | AAACNAT.G. | TGT.GAACCT TTTTTT.AAA A.TAGA..GG |
| N30199 | .GGGAGGNG. | AGACTAT.G. | TGT.AA.GCT TTTTTT..AA A.TAGA..GG |
| T52966 | | | |
| N29508 | .GGGAGGGG. | AGACTA..G. | TGT.GA.GCT TTTTTT..AA A.TAGA..GG |
| N26919 | | | |
| N26910 | | | |
| H16757 | | | |
| N27732 | | | |
| | 1251 | | 1300 |
| Bikunin | GATTGACTC | GGATTTC A | GT GATC A TTAGGG CT GAGGTCTGTT |
| R32676 | GNITGANTTC | GGGNTTTTNA | GTGATCCAT TTAGGGGGNT GAG |
| T47439 | GATTGACTC. | .GGATTTC.A | GT.GATC.A. TTAGGG..CT GAGGTCTNTT |
| R73968 | GATTGACTC. | .GGATTTC.A | GT.GATC.A. TTAGGG..CT GAGGTCTGTT |
| H39840 | GATTGACTC. | .GGATTTC.A | GT.GATC.A. TTAGGG..CT GAGGTCTGTT |
| H95233 | |A. | TTAGGG..CT GAGGTCTGTT |
| H39841 | GATTGACTC. | .GGATTTC.A | GT.GATC.A. TTAGGG..CT GAGGTCTGTT |
| N30199 | GATTGACTC. | .GGATTTCGA | GT.GATC.A. TTAGGG..CT GAGGTCTGTT |
| T52966 | | | |
| N29508 | GATTGACTC. | .GGATTTC.A | GT.GATCNA. TTAGGG..CT GAGGTCTGTT |
| N26919 | | | |
| N26910 | | | |
| H16757 | | | |
| N27732 | | | |
| | 1301 | | 1350 |
| Bikunin | TCTCTGGGAG | GTAGGACGGC | TGCTTCC TG G TC TGGCA GGGATGGG |
| T47439 | TCTCTNGGAG | GTAGGACGA | |
| R73968 | TCTCTGGGAG | GTAGGACGGC | TGCTTCC.TG GGTCTTGGCA .GGGATGGG |
| H39840 | TCTCTGGGAG | GTAGGACGGC | TGCTTCC.TG G.TC.TGGCA .GGGATGGG. |
| H95233 | NCTCTGGGAG | NTAGGACGGC | TGCTTCCCTG G.TC.TGGCA .GGGATGGG. |
| H39841 | TCNCTGGGAG | GTAGGACGGC | TGCTTCCCTG G.TC.TGGCA .GGGATGGG. |
| N30199 | TCTCTGGGAG | GTAGGACGGC | TGCTTCC.TG G.TC.TGGCA .GGGATGGG. |
| T52966 | | | ..TC.TGGCA .GGGATGGG. |
| N29508 | TCTCTGGGAG | GTAGGACGGC | TGCTTCA.TG G.TC.TGGCA .GGGATGGG. |
| N26919 | | | |
| N26910 | | | |
| H16757 | |G | G.TC.TGGCA .GGGATGGG. |
| N27732 | |CCCTG | GGTCTGNCA ACGNATGGG |

25/41

Figure 4C (Con't)

| | | | |
|---------|--|--|------|
| | 1351 | | 1400 |
| Bikunin | TTTG CTTTG G AAATCCTC T AGGAGGCT CCTCCT CGC ATGG CC TG | | |
| R73968 | TTTG.CTTTG GGAATCCTC TTNGGAGGCT CCTCCTTCGC ATGGGCCCTTG | | |
| H39840 | TTTG.CTTTG GAGAATCCTC T.ANGAGGCT CCTCCT.CGC ATGG.CC.TG | | |
| H95233 | TTTG.CTTTG G.AAATCCTC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG | | |
| H39841 | TTTG.CTTTG G.AAANCCNC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG | | |
| N30199 | TTTG.CTTTG G.AAATCCTC T.AGGAGGCT CCTCCTTCGC ATGG.CC.TG | | |
| T52966 | TTTG.CTTTG G.AAATCCTC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG | | |
| N29508 | TTTG.CTTTG G.AAATCCTC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG | | |
| N26919 | GAGGCT CCTCCT.CGC ATGG.CC.TG | | |
| N26910 |CTTTT GNAATCCTC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG | | |
| H16757 | TTTGCCCTTG G.AAANCCTC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG | | |
| N27732 | TTTG.CTTTG G.AAATCCTC TTAGGAGGCT CCTCCT.CGC ATGG.CC.TG | | |
| | 1401 | | 1450 |
| Bikunin | CAGT CT GG CAGCAG CCC CGAGTTGTTT CC TCGCTG ATC GATTTC | | |
| R73968 | CAGT.CTNGG CAGCANCCCC CGAGTTTTTT TCCTTCGCTG ATCCGATTTC | | |
| H39840 | CAGT.CT.GG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC | | |
| H95233 | CAGTTCT..G CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC | | |
| H39841 | CAGT.CT.GG CAGCAG.CCC CGAGTTGTTN .CC.TCGCTG ATC.GATNTC | | |
| N30199 | CAGT.CT.GG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC | | |
| T52966 | CAGT.CT.GG CAGCAG..CC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC | | |
| N29508 | CAGT.CT..G CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC | | |
| N26919 | CAGT.CTTGG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ANC.GATTTC | | |
| N26910 | CAGT.CT..G CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATCGGATTTC | | |
| H16757 | CAGTNCT.GG CAGCAGACCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC | | |
| N27732 | CAGT.CT.GG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ANC.GATTTC | | |
| | 1451 | | 1500 |
| Bikunin | TTT CCTCCA GGTAG AGT TTTC TTG CTTATGTTGA ATTCCATTGC | | |
| R73968 | TTTTCTCCA GGTAAGAATT TTTCTTTT | | |
| H39840 | TTT.CCTCCA GGTAG..AGT TTTC.TTG. CTTATGTTGA ATTCCATTGC | | |
| H95233 | TTT.CCTCCA GGTAG..AGT TTTC.TTG. CTTATGTTGA ATTCCATTGC | | |
| H39841 | TTT.CCCCCA GGTAG..AGT TTTC.TTG. CTTATGTTGA ANTCCATTGC | | |
| N30199 | TTT.CCTCCA GGTAG..AGT TTTC.TTG. CTTATGTTGA ATTCCATTGC | | |
| T52966 | TTT.CCTCCA GGTAG..AGT TTTC.TTG. CTTATGTTGA ATTCCATTGC | | |
| N29508 | TTT.CCTCCA GGTAG..AGT TTTC.TTG. CTTATGTTGA ATTCCATTGC | | |
| N26919 | TTT.CCNCCA GGTAG..AGT TTTC.TTG. CTTATGTTGA ATTCCATTGC | | |
| N26910 | TTT.CCTCCA GGTAG..AGT TTTC.TTG. CTTATGTTGA ATTCCATTGC | | |
| H16757 | TTTACCCCCA GGTAG..AGT TTTCCTTGN CTTATGTTGA ATTCCATTGC | | |
| N27732 | TTT.CCTCCA GGTAG..AGT TTTC.TTG. CTTATGTTGA ATTCCATTGC | | |

26/41

Figure 4C (Con't)

1501 1550
 Bikunin CTCTTTT CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT GT
 H39840 CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTTGT
 H95233 CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
 H39841 CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
 N30199 CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
 T52966 CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
 N29508 CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
 N26919 CTCTTTT.CN CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
 N26910 CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
 H16757 CTCTTTTACT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
 N27732 CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT

1551 1600
 Bikunin CTGATTATG G TTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
 H39840 CTGATTATG GTTTTTTTT AAGTAT
 H95233 CTGATTATG G..TTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
 H39841 CTGATTATG G..TTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
 N30199 CTGATTATG G..TTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
 T52966 CTGATTATG G..TTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
 N29508 CTGATTATG G..TTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
 N26919 CTGATTATG G..TTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
 N26910 CTGATTATG G..TTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
 H16757 CTGATTATG G..TTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
 N27732 CTGATTATG G..TTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT

1601 1650
 Bikunin CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
 H95233 CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAA
 H39841 CTGAAAGAAG GAAAGTAAAN TGTACAAGTT TAATAAAAAG GGGCCTTCCC
 N30199 CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
 T52966 CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
 N29508 CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
 N26919 CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
 N26910 CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
 H16757 CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
 N27732 CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC

1651 1689
 Bikunin CTTTAG AAT AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA
 H39841 CTTTAA.
 N30199 CTTTAG.AAT AAA
 T52966 CTTTAGGAAT NAAAAA AAAAGGTG
 N29508 CTTTAG.AAT AAATTCAGC ATGTGCTTTC AA
 N26919 CTTTAG.AAT AAAAAAAAAA AAAAAAAAAA A
 N26910 CTTTAG.AAT AAATTCAGC ATGTGCTTTC AAAAAA
 H16757 CTTTAG.AAT AAAAAAAAAA AAAAAAAAAA AAAAAA
 N27732 CTTTAG.AAT AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA

27/41

FIGURE 4D

EST consensus MLRAEADGVS RLLGSLLLLSG VLAADREESI HDFCLVSKVV GRCRASMPRW 50
EST consensus WYNVTDGSCQ LFVYGGCDGN SNNYLTKEEC LKKCATVTEN ATGDLATSRN 100
EST consensus AADSSVPSAP RRQDSEDHSS DMFNYYEYCT ANAVTGPCRA SFPRWYFDVE 150
EST consensus RNSCNNFIYG GCRGNKNSYR SEEACMLRCF RQENPPLPL GSKVVVLAGL 200
EST consensus FVMVLILELG ASMVYLIRVA RRNQERALRT VWSSGDDKEQ LVKNITYVL 248

FIGURE 4E

cDNA ACC 3
translation T -47

cDNA TGATCGCGAG ACCCCAACGG CTGGTGGCGT CGCCTGCGCG TCTCGGCTGA 53
translation . S R D P N G W W R R L R V S A E -30

cDNA GCTGGCCATG GCGCAGCTGT GCGGGCTGAG GCGGAGCCGG GCGTTTCTCG 103
translation L A M A Q L C G L R R S R A F L A-13

cDNA CCCTGCTGGG ATCGCTGCTC CTCTCTGGGG TCCTGGCGGC CGACCGAGAA 153
translation L L G S L L L S G V L A A D R E 4

cDNA CGCAGCATCC ACGACTTCTG CCTGGTGTCTG AAGGTGGTGG GCAGATGCCG 203
translation R S I H D F C L V S K V V G R C R 21

cDNA GGCTCCATG CCTAGGTGGT GGTACAATGT CACTGACGGA TCCTGCCAGC 253
translation A S M P R W W Y N V T D G S C Q L 38

cDNA TGT TTGTGTA TGGGGGCTGT GACGGAAACA GCAATAATTA CCTGACCAAG 303
translation F V Y G G C D G N S N N Y L T K 54

cDNA GAGGAGTGCC TCAAGAAATG TGCCACTGTC ACAGAGAATG CCACGGGTGA 353
translation E E C L K K C A T V T E N A T G D 71

cDNA CCTGGCCACC AGCAGGAATG CAGCGGATTC CTCTGTCCCA AGTGCTCCCA 403
translation L A T S R N A A D S S V P S A P R 88

cDNA GAAGGCAGGA TTCTGAAGAC CACTCCAGCG ATATGTTCAA CTATGAAGAA 453
translation R Q D S E D H S S D M F N Y E E 104

cDNA TACTGCACCG CCAACGCAGT CACTGGGCCT TGCCGTGCAT CCTTCCCACG 503
translation Y C T A N A V T G P C R A S F P R 121

cDNA CTGGTACTTT GACGTGGAGA GGAACCTCTG CAATAACTTC ATCTATGGAG 553
translation W Y F D V E R N S C N N F I Y G G 138

cDNA GCTGCCGGGG CAATAAGAAC AGCTACCGCT CTGAGGAGGC CTGCATGCTC 603
translation C R G N K N S Y R S E E A C M L 154

cDNA CGCTGCTTCC GCCAGCAGGA GAATCCTCCC CTGCCCCTTG GCTCAAAGGT 653
translation R C F R Q Q E N P P L P L G S K Y 171

cDNA GGTGGTTCCTG GCGGGGCTGT TCGTGATGGT GTTGATCCTC TTCCTGGGAG 703
translation V V L A G L F V M V L I L F L G A 188

cDNA CCTCCATGGT CTACCTGATC CGGGTGGCAC GGAGGAACCA GGAGCGTGCC 753
translation S M V Y L I R V A R R N Q E R A 204

cDNA CTGCGCACCG TCTGGAGCTT CGGAGATGA 782
translation L R T V W S F G D 213

FIGURE 4F

```

cDNA      GCACGAGTTG GGAGGTGTAG CGCGGCTCTG AACGCGCTGA GGGCCGTGA 50
cDNA      GTGTCGCAGG CGGCGAGGGC GCGAGTGAGG AGCAGACCCA GGCATCGCGC 100
cDNA      GCCGAGAAGG CCGGGCGTCC CCACACTGAA GGTCCGGAAA GGC GACTTCC 150
cDNA      GGGGGCTTTG GCACCTGGCG GACCCTCCCG GAGCGTCGGC ACCTGAACGC 200
cDNA      GAGGCGCTCC ATTGCGCGTG CGCGTTGAGG GGCTTCCCGC ACCTGATCGC 250
cDNA      GAGACCCCAA CGGCTGGTGG CGTCGCCTGC GCGTCTCGGC TGAGCTGGCC 300
cDNA      ATGGCGCAGC TGTGCGGGCT GAGGCGGAGC CGGGCGTTTC TCGCCCTGCT 350
translation M A Q L C G L R R S R A F L A L L -11

cDNA      GGGATCGCTG CTCCTCTCTG GGGTCTCTGGC GGCCGACCGA GAACGCAGCA 400
translation G S L L L S G V L A A D R E R S I 7

cDNA      TCCACGACTT CTGCCTGGTG TCGAAGGTGG TGGGCAGATG CCGGGCCTCC 450
translation H D F C L V S K V V G R C R A S 23

cDNA      ATGCCTAGGT GGTGGTACAA TGTCAGTAC GGATCCTGCC AGCTGTTTGT 500
translation M P R W W Y N V T D G S C Q L F V 40

cDNA      GTATGGGGGC TGTGACGGAA ACAGCAATAA TTACCTGACC AAGGAGGAGT 550
translation Y G G C D G N S N N Y L T K E E C 57

cDNA      GCCTCAAGAA ATGTGCCACT GTCACAGAGA ATGCCACGGG TGACCTGGCC 600
translation L K K C A T V T E N A T G D L A 73

cDNA      ACCAGCAGGA ATGCAGCGGA TTCCTCTGTC CCAAGTGCTC CCAGAAGGCA 650
translation T S R N A A D S S V P S A P R R Q 90

cDNA      GGATTCTGAA GACCACTCCA GCGATATGTT CAACTATGAA GAATACTGCA 700
translation D S E D H S S D M F N Y E E Y C T 107

cDNA      CCGCCAACGC AGTCACTGGG CCTTGCCGTG CATCCTTCCC ACGCTGGTAC 750
translation A N A V T G P C R A S F P R W Y 123

cDNA      TTTGACGTGG AGAGGAACTC CTGCAATAAC TTCATCTATG GAGGCTGCCG 800
translation F D V E R N S C N N F I Y G G C R 140

cDNA      GGGCAATAAG AACAGCTACC GCTCTGAGGA GGCCTGCATG CTCCGCTGCT 850
translation G N K N S Y R S E E A C M L R C F 157

cDNA      TCCGCCAGCA GGAGAATCCT CCCCTGCCCC TTGGCTCAAA GGTGGTGGTT 900
translation R Q Q E N P P L P L G S K V V V 173

cDNA      CTGGCGGGGC TGTTCGTGAT GGTGTTGATC CTCTTCCTGG GAGCCTCCAT 950
translation L A G L F V M V L I L F L G A S M 190

cDNA      GGTCTACCTG ATCCGGGTGG CACGGAGGAA CCAGGAGCGT GCCCTGCGCA 1000
translation V Y L I R V A R R N Q E R A L R T 207

cDNA      CCGTCTGGAG CTCCGGAGAT GACAAGGAGC AGCTGGTGAA GAACACATAT 1050
translation V W S S G D D K E Q L V K N T Y 223

cDNA      GTCCTGTGAC CGCCCTGTCG CCAAGAGGAC TGGGGAAGGG AGGGGAGACT 1100
translation V L * 225

```

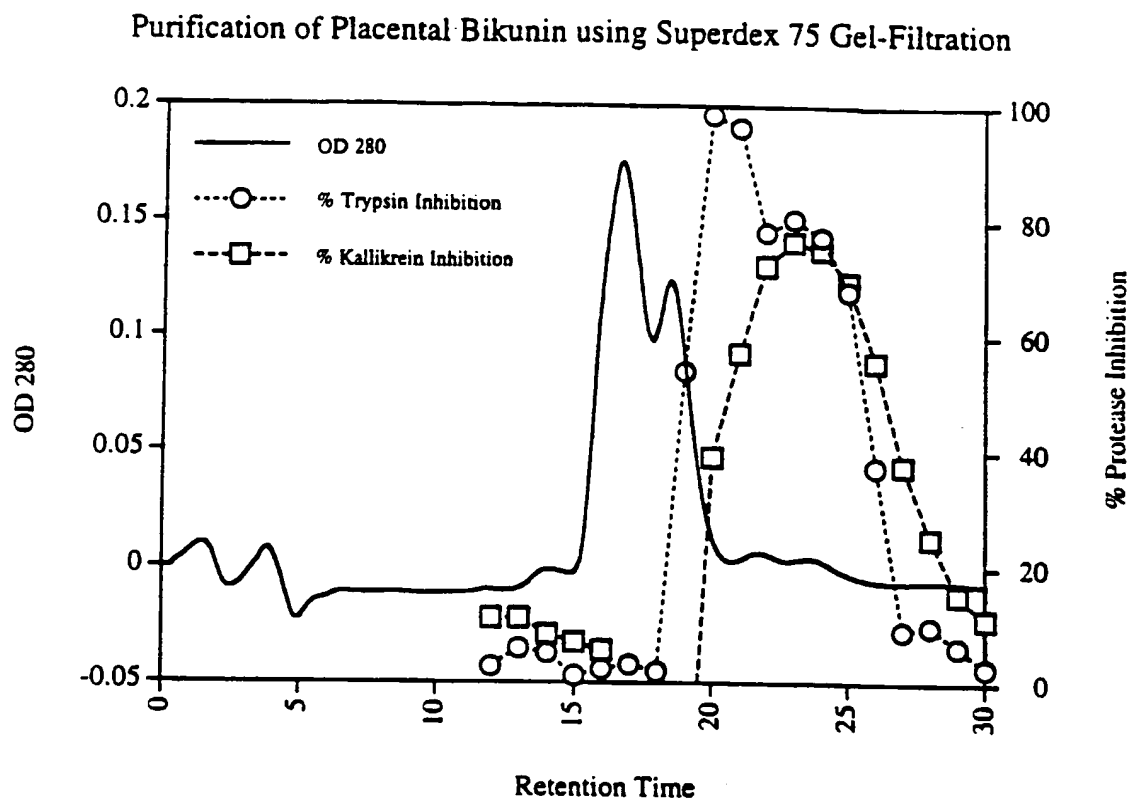
FIGURE 4F (Con't)

| | | | | | | |
|------|------------|------------|------------|------------|------------|------|
| cDNA | ATGTGTGAGC | TTTTTTTAAA | TAGAGGGATT | GACTCGGATT | TGAGTGATCA | 1150 |
| cDNA | TTAGGGCTGA | GGTCTGTTTC | TCTGGGAGGT | AGGACGGCTG | CTTCCTGGTC | 1200 |
| cDNA | TGGCAGGGAT | GGGTTTGCTT | TGGAAATCCT | CTAGGAGGCT | CCTCCTCGCA | 1250 |
| cDNA | TGGCCTGCAG | TCTGGCAGCA | GCCCCGAGTT | GTTTCCTCGC | TGATCGATTT | 1300 |
| cDNA | CTTTCCTCCA | GGTAGAGTTT | TCTTTGCTTA | TGTTGAATTC | CATTGCCTCC | 1350 |
| cDNA | TTTTCTCNAT | CACAGAAGTG | ATGTTGGAAT | CGTTTCTTTT | GTTTGTCTGA | 1400 |
| cDNA | TTTATGGTTT | TTTTAAGTAT | AAACAAAAGT | TTTTTATTAG | CATTCTGAAA | 1450 |
| cDNA | GAAGGAAAGT | AAAATGTACA | AGTTTAATAA | AAAGGGGCCT | TCCCCTTTAG | 1500 |
| cDNA | AATAAATTTC | CAGCATGTTG | CTTTCAAAAA | AAAAAAAAAA | AAAA | |

1550

FIGURE 4G

| | | | | |
|---------------|------------|------------|------------------|-------------------|
| EST consensus | MLR | AEADGVSRL | GSLLLSGVLA | -1 |
| PCR clone | MAQLCGL | RRSRAFLALL | GSLLLSGVLA | -1 |
| λcDNA clone | MAQLCGL | RRSRAFLALL | GSLLLSGVLA | -1 |
| | | | | |
| EST consensus | ADRERSIHDF | CLVSKVVGRC | RASMPRWYIN | VTDGSCQLFV |
| PCR clone | ADRERSIHDF | CLVSKVVGRC | RASMPRWYIN | VTDGSCQLFV |
| λcDNA clone | ADRERSIHDF | CLVSKVVGRC | RASMPRWYIN | VTDGSCQLFV |
| | | | | |
| EST consensus | YLTKEECLKK | CATVTENATG | DLATSRNAAD | SSVPSAPRRQ |
| PCR clone | YLTKEECLKK | CATVTENATG | DLATSRNAAD | SSVPSAPRRQ |
| λcDNA clone | YLTKEECLKK | CATVTENATG | DLATSRNAAD | SSVPSAPRRQ |
| | | | | |
| EST consensus | NYEEYCTANA | VTGPCRASFP | RWYFDVERNS | CNNFIYGGCR |
| PCR clone | NYEEYCTANA | VTGPCRASFP | RWYFDVERNS | CNNFIYGGCR |
| λcDNA clone | NYEEYCTANA | VTGPCRASFP | RWYFDVERNS | CNNFIYGGCR |
| | | | | |
| EST consensus | ACMLRCFRQQ | ENPPLPLGSK | <u>VVVLAGLEV</u> | <u>VLILFLGASM</u> |
| PCR clone | ACMLRCFRQQ | ENPPLPLGSK | <u>VVVLAGLEV</u> | <u>VLILFLGASM</u> |
| λcDNA clone | ACMLRCFRQQ | ENPPLPLGSK | <u>VVVLAGLEV</u> | <u>VLILFLGASM</u> |
| | | | | |
| EST consensus | QERALRTVWS | SGDDKEQLVK | NTYVL | 225 |
| PCR clone | QERALRTVWS | FGD | | 213 |
| λcDNA clone | QERALRTVWS | SGDDKEQLVK | NTYVL | 225 |

**FIGURE 5**

33/41

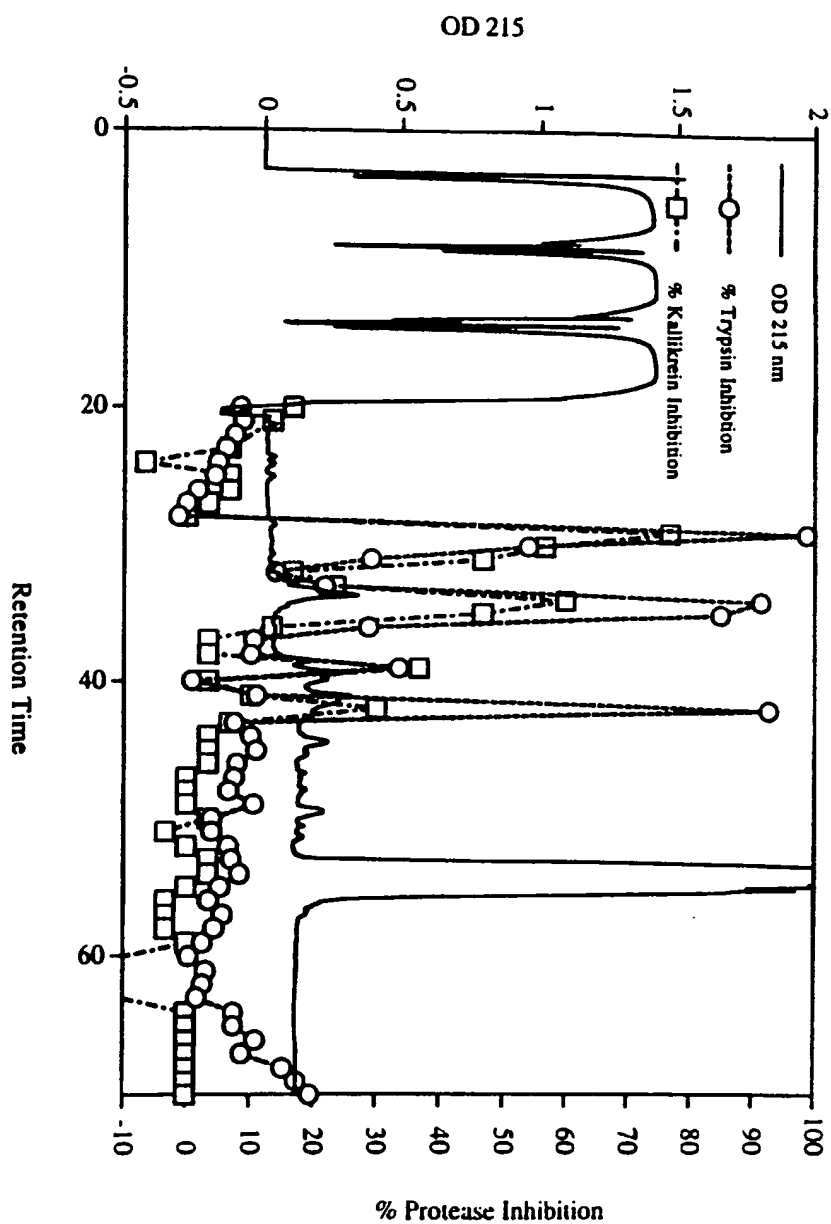


FIGURE 6

Purification of Placental Bikunin using C18 Reverse-Phase Chromatography

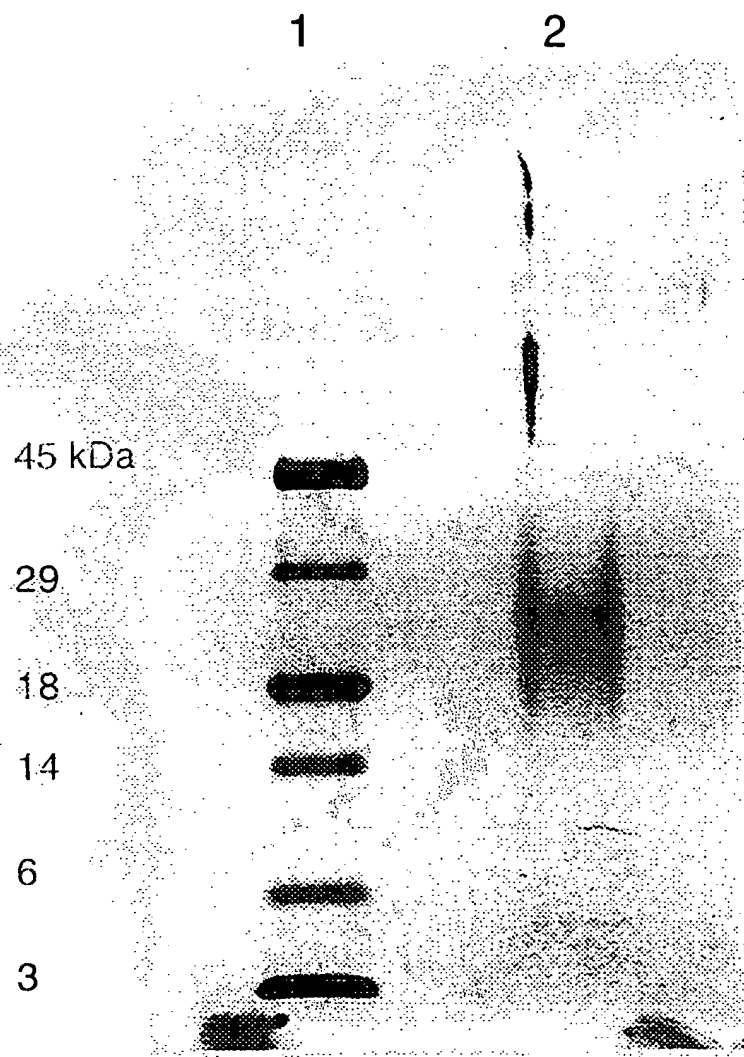
Figure 7

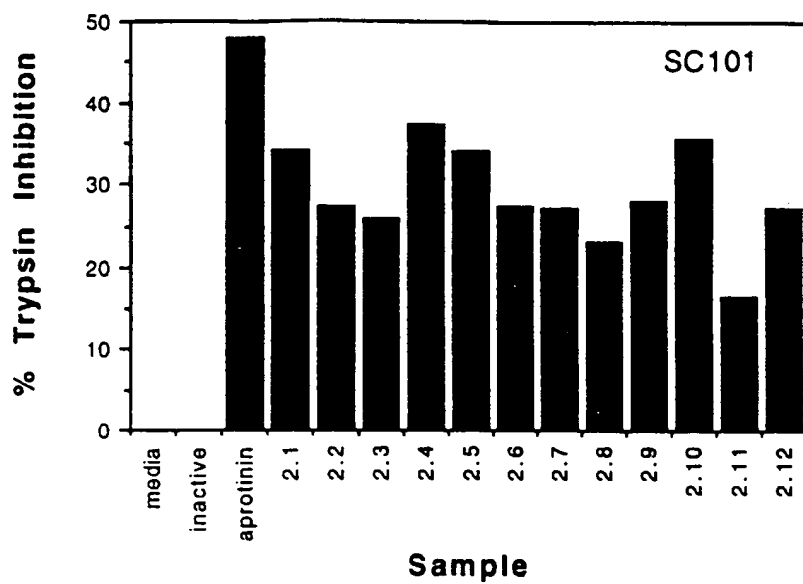
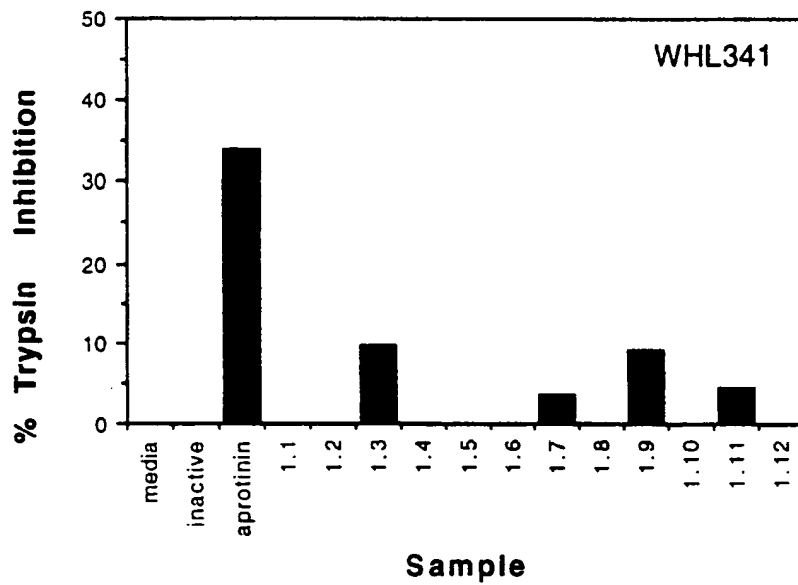
Figure 8A**Figure 8B**

Figure 9B

Western

Aprotinin
2.5
2.4

45 kDa
29
18
14
6
3

Figure 9A

SDS-PAGE

Aprotinin
2.5
2.4

45 kDa
29
18
14
6
3

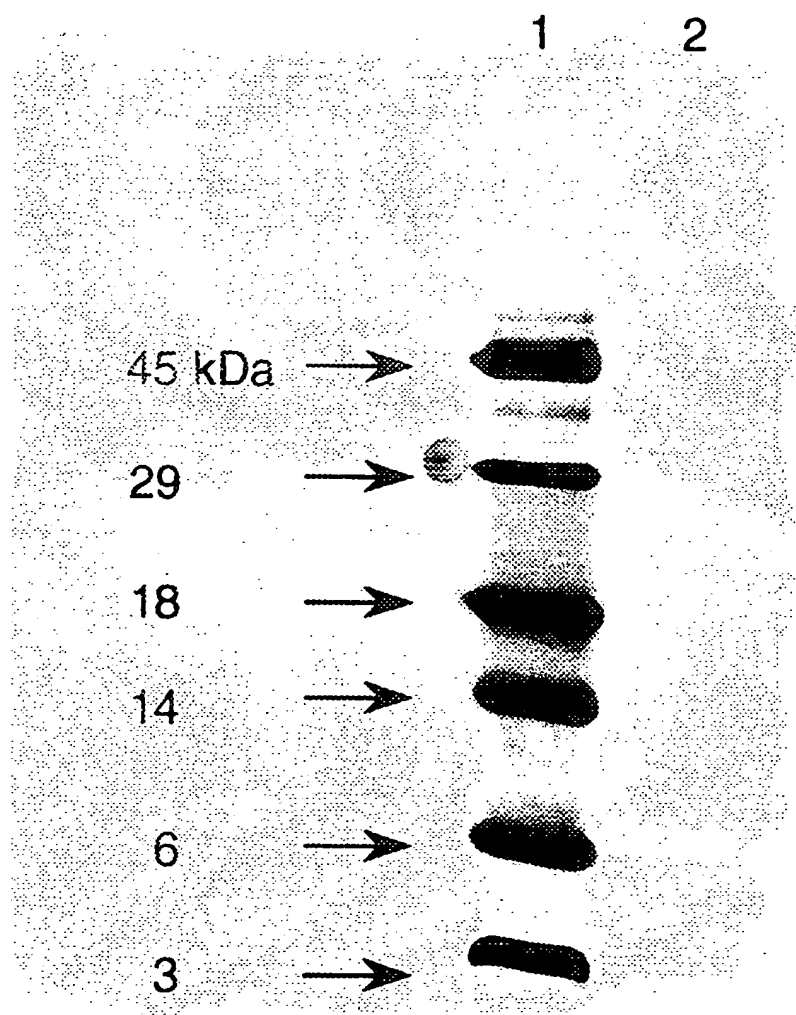
Figure 10

Figure 11A

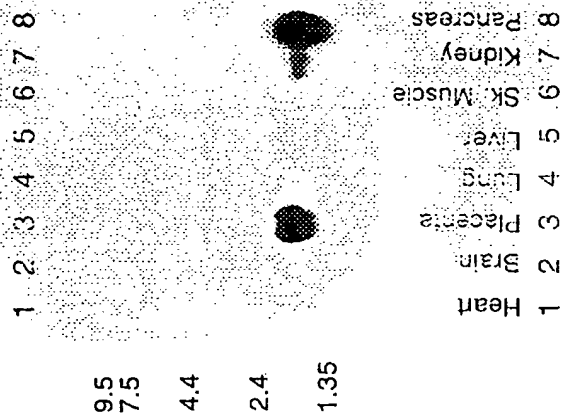


Figure 11B

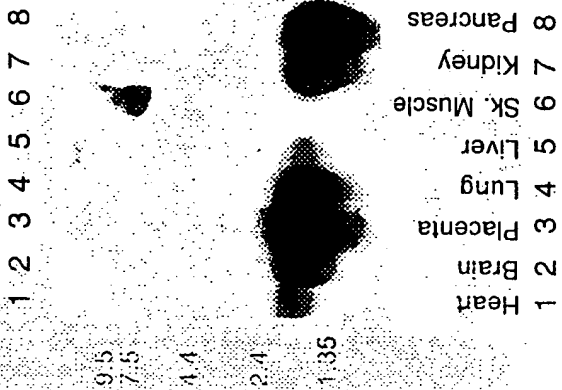


Figure 12A

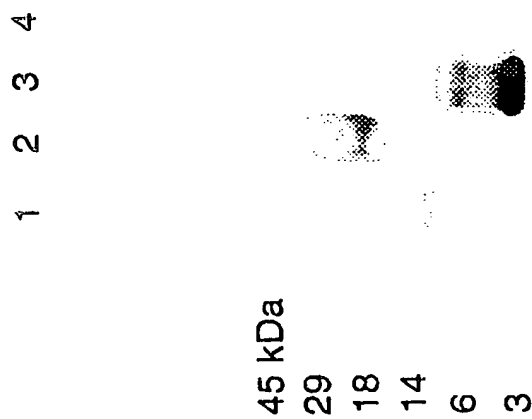


Figure 12B

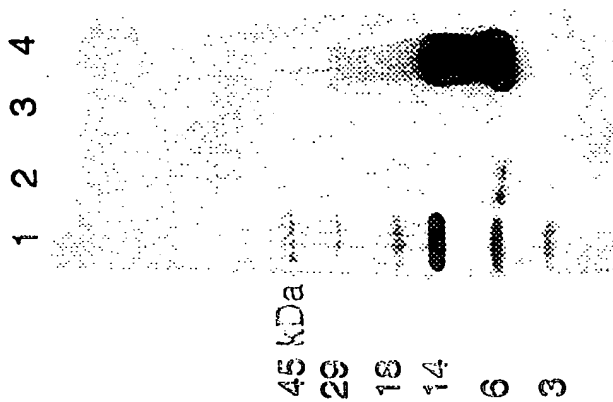


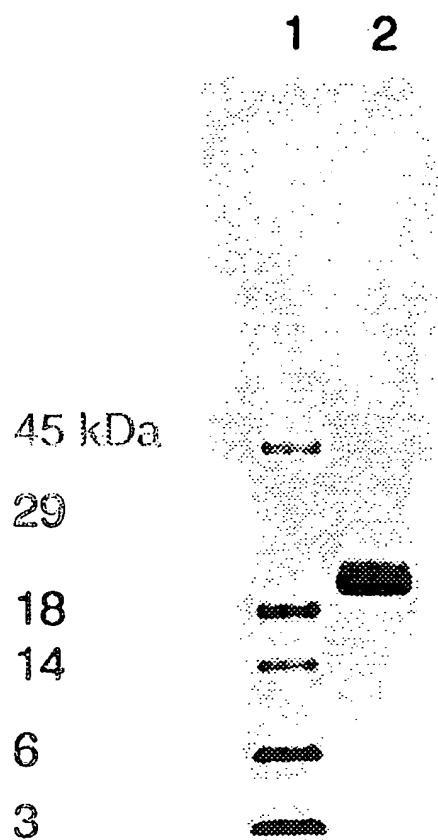
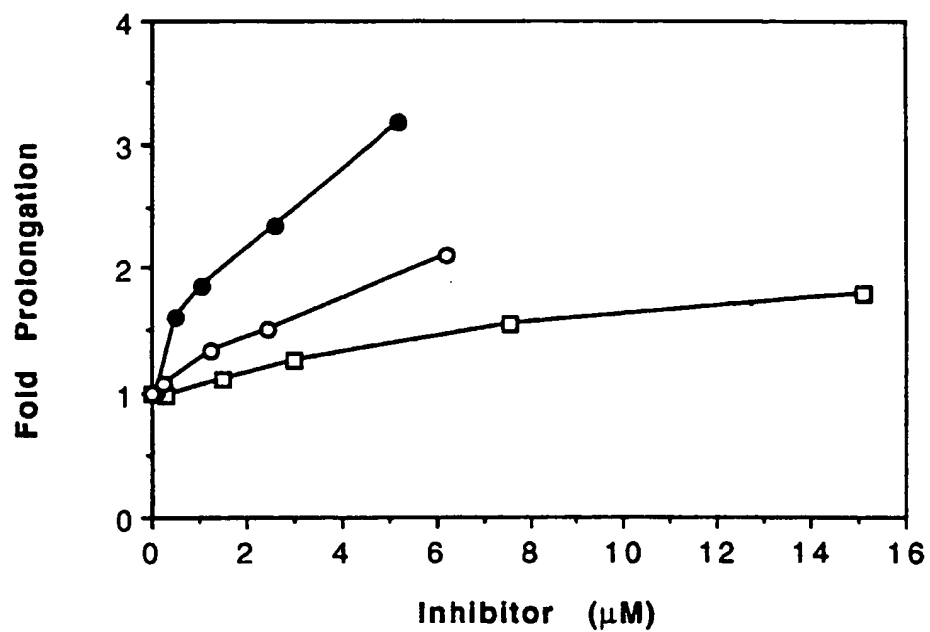
Figure 13

Figure 14



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/03894

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/15 C07K14/81 A61K38/57

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|-----------------------|
| X | EMBL/GENBANK DATABASES Accession no R35464 Sequence reference HS46499, May 4, 1995 L.HILLIER ET AL: "The WashU-Merck EST Project" XP002039653 see the whole document --- | 1-6,11 |
| X | EMBL/GENBANK DATABASES Accession no N39798 Sequence reference HS798277, January 26, 1996 L. HILLIER ET AL: "The WasU-Merck EST Project" XP002039654 see the whole document --- -/-- | 1-6,11 |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

4 September 1997

Date of mailing of the international search report

01.10.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Van der Schaal, C

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/03894

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|-----------------------|
| X | EMBL/GENBANK DATABASES Accession no R74593 Sequence reference HS593137, June 9, 1995 L.HILLIER ET AL: "The WashU-Merck EST Project" XP002039655 see the whole document --- | 1-6,11 |
| P,X | EP 0 758 682 A (MITSUBISHI CHEM CORP) 19 February 1997 see the whole document --- | 1-11 |
| P,A | JOURNAL OF BIOLOGICAL CHEMISTRY 272 (10). 1997. 6370-6376. ISSN: 0021-9258, XP002039700 SHIMOMURA T ET AL: "Hepatocyte growth factor activator inhibitor, a novel Kunitz-type serine protease inhibitor." --- | |
| A | DATABASE MEDLINE accession no 94289695, 1 July 1994 J. WOJTA ET AL: "Hepatocyte growth factor stimulates expression of plasminogen activator inhibitor type 1 and tissue factor in HepG2 cells" XP002039702 see abstract & BLOOD, vol. 84, no. 1, 1994, pages 151-157, --- | 7-10 |
| A | JOURNAL OF BIOLOGICAL CHEMISTRY 271 (7). 1996. 3615-3618. ISSN: 0021-9258, XP002039701 MIYAZAWA K ET AL: "Activation of hepatocyte growth factor in the injured tissues is mediated by hepatocyte growth factor activator." ----- | 7-10 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 97/03894

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 7-9
is(are) directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

information to patent family members

PCT/US 97/03894

Form PCT/ISA/210 (patent family annex) (July 1992)

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER: _____**

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
22 July 2004 (22.07.2004)

PCT

(10) International Publication Number
WO 2004/060403 A2

(51) International Patent Classification⁷: **A61K 47/48**,
A61P 25/00

(21) International Application Number:
PCT/CA2004/000011

(22) International Filing Date: 5 January 2004 (05.01.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/437,986 6 January 2003 (06.01.2003) US

(71) Applicant (for all designated States except US): **TRANS-
FERT PLUS** [CA/CA]; 550 Sherbrooke West, Suite 100,
H3A 1B9 Montréal, Québec (CA).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **BÉLIVEAU,**
Richard [CA/CA]; 266 Wilson, H3E 1L8 Montréal,
Québec, CA (CA). **DEMEULE, Michel** [CA/CA]; 3557
Archambault, J4M 2W8 Longueuil, Québec, CA (CA).

(74) Agent: **OGILVY RENAULT**; Suite 1600, 1981 McGill
College Avenue, H3A 2Y3 Montréal, Québec, CA (CA).

(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD,
MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG,
PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM,
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM,
ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), Euro-
pean (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR,
GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished
upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: A METHOD FOR TRANSPORTING A COMPOUND ACROSS THE BLOOD-BRAIN BARRIER

(57) Abstract: The present invention relates to improvements in the field of drug delivery. More particularly, the invention relates to a non-invasive and flexible method and carrier for transporting a compound or drug across the blood-brain barrier of an individual. In particular the present invention relates to a carrier for transporting an agent attached thereto across a blood-brain barrier, wherein the carrier is able to cross the blood-brain barrier after attachment to the agent and thereby transport the agent across the blood-brain barrier. The present invention relates to improvements in the field of drug delivery. More particularly, the invention relates to a non-invasive and flexible method and carrier for transporting a compound or drug across the blood-brain barrier of an individual. In particular the present invention relates to a carrier for transporting an agent attached thereto across a blood-brain barrier, wherein the carrier is able to cross the blood-brain barrier after attachment to the agent and thereby transport the agent across the blood-brain barrier.

BEST AVAILABLE COPY

A METHOD FOR TRANSPORTING A COMPOUND ACROSS THE BLOOD-BRAIN BARRIER

TECHNICAL FIELD

[0001] The present invention relates to improvements in the field of drug delivery. More particularly, the invention relates to a non-invasive and flexible method and carrier for transporting a compound or drug across the blood-brain barrier of an individual.

BACKGROUND OF THE INVENTION

[0002] In the development of a new therapy for brain pathologies, the blood-brain barrier (BBB) is considered as a major obstacle for the potential use of drugs for treating disorders of the central nervous system (CNS). The global market for CNS drugs was \$33 billion in 1998, which was roughly half that of global market for cardiovascular drugs, even though in the United States, nearly twice as many people suffer from CNS disorders as from cardiovascular diseases. The reason for this lopsidedness is that more than 98% of all potential CNS drugs do not cross the blood-brain barrier. In addition, more than 99% of worldwide CNS drug development is devoted solely to CNS drug discovery, and less than 1% is directed to CNS drug delivery. This ratio could justify why no efficient treatment is currently available for the major neurological diseases such as brain tumors, Alzheimer's and stroke.

[0003] The brain is shielded against potentially toxic substances by the presence of two barrier systems: the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB). The BBB is considered to be the major route for the uptake of serum ligands since its surface area is approximately 5000-fold greater than that of BCSFB. The brain endothelium, which constitutes the BBB, represents the major obstacle for the use of potential drugs against many disorders of the CNS. As a general rule, only lipophilic molecules smaller than about 500 Daltons can pass

- 2 -

across the BBB, i.e., from blood to brain. However, the size of many drugs that show promising results in animal studies for treating CNS disorders is considerably bigger. Thus, peptide and protein therapeutics are generally excluded from transport from blood to brain, owing to the negligible permeability of the brain capillary endothelial wall to these drugs. Brain capillary endothelial cells (BCECs) are closely sealed by tight junctions, possess few fenestrae and few endocytic vesicles as compared to capillaries of other organs. BCECs are surrounded by extracellular matrix, astrocytes, pericytes and microglial cells. The close association of endothelial cells with the astrocyte foot processes and the basement membrane of capillaries are important for the development and maintenance of the BBB properties that permit tight control of blood-brain exchange.

[0004] To date, there is no efficient drug delivery approach available for the brain. The methods under investigation for peptide and protein drug delivery to the brain may be divided in three principal strategies. Firstly, invasive procedures include the direct intraventricular administration of drugs by means of surgery, and the temporary disruption of the BBB via intracarotid infusion of hyperosmolar solutions. Secondly, the pharmacologically-based strategy consists in facilitating the passage through the BBB by increasing the lipid solubility of peptides or proteins. Thirdly, physiologic-based strategies exploit the various carrier mechanisms at the BBB, which have been characterized in the recent years. In this approach, drugs are attached to a protein vector that performs like receptors-targeted delivery vehicle on the BBB. This approach is highly specific and presents high efficacy with an extreme flexibility for clinical indications with unlimited targets. In the present invention, the latter approach has been investigated.

[0005] It would be highly desirable to be provided with an improvement in the field of drug delivery.

[0006] It would also be highly desirable to be provided with a non-invasive and flexible method and a carrier for transporting a compound or drug across the BBB of an individual.

SUMMARY OF THE INVENTION

[0007] One aim of the present invention is to provide an improvement in the field of drug delivery.

[0008] Another aim of the present invention is to provide a non-invasive and flexible method and carrier for transporting a compound or drug across the blood-brain barrier of an individual.

[0009] According to one embodiment of the invention, there is provided a method for transporting an agent across the blood-brain barrier of a patient, which comprises the step of administering to the patient a compound comprising the agent attached to aprotinin, a pharmaceutically acceptable salt of aprotinin, a fragment of aprotinin or a pharmaceutically acceptable salt of a fragment of aprotinin.

[0010] According to a further embodiment of the invention, there is provided a use of aprotinin, a pharmaceutically acceptable salt of aprotinin, a fragment of aprotinin or a pharmaceutically acceptable salt of a fragment of aprotinin for transporting a compound attached thereto across the blood-brain barrier of a patient.

[0011] According to another embodiment of the invention, there is provided a use of aprotinin, a pharmaceutically acceptable salt of aprotinin, a fragment of aprotinin or a pharmaceutically acceptable salt of a fragment of aprotinin in the manufacture of a medicament for treating a neurological disease across the blood-brain barrier of a patient.

[0012] According to yet another embodiment of the invention, there is provided a use of aprotinin, a pharmaceutically acceptable salt of aprotinin,

a fragment of aprotinin or a pharmaceutically acceptable salt of a fragment of aprotinin in the manufacture of a medicament for treating a central nervous system disorder across the blood-brain barrier of a patient.

[0013] According to another embodiment of the invention, there is provided compounds of formula R-L-M or pharmaceutically acceptable salts thereof, wherein R is aprotinin or a fragment thereof, L is a linker or a bond and M is an agent or a drug selected from the group consisting of a small molecule drug, a protein, a peptide and an enzyme.

[0014] According to another embodiment of the invention, there is provided a method for treating a neurological disease of a patient comprising administering to the patient a medicament comprising aprotinin, a pharmaceutically acceptable salt of aprotinin, a fragment of aprotinin or a pharmaceutically acceptable salt of a fragment of aprotinin, and a compound adapted to treat the disease, the compound being attached to the aprotinin.

[0015] According to a further embodiment of the invention, there is provided a method for treating a central nervous system disorder of a patient comprising administering to the patient a medicament comprising aprotinin, a pharmaceutically acceptable salt of aprotinin, a fragment of aprotinin or a pharmaceutically acceptable salt of a fragment of aprotinin, and a compound adapted to treat the disease, the compound being attached to the aprotinin.

[0016] In accordance with one embodiment of the present invention, there is provided a carrier for transporting an agent attached thereto across a blood-brain barrier, wherein the carrier is able to cross the blood-brain barrier after attachment to the agent and thereby transport the agent across the blood-brain barrier.

[0017] In a preferred embodiment of the present invention, the transporting does not affect blood-brain barrier integrity.

[0018] In a preferred embodiment of the present invention, the carrier is selected from the group consisting of aprotinin, a functional derivative of aprotinin, Angio-pep1 and a functional derivative of Angio-pep1.

[0019] In a preferred embodiment of the present invention, the agent is selected from the group consisting of a drug, a medicine, a protein, a peptide, an enzyme, an antibiotic, an anti-cancer agent, a molecule active at the level of the central nervous system, a radioimaging agent, an antibody, a cellular toxin, a detectable label and an anti-angiogenic compound.

[0020] In a preferred embodiment of the present invention, the anti-cancer agent is Paclitaxel.

[0021] In a preferred embodiment of the present invention, the detectable label is selected from the group consisting of a radioactive label, a green fluorescent protein, a histag protein and β -galactosidase.

[0022] In a preferred embodiment of the present invention, the agent has a maximum molecular weight of 160,000 Daltons.

[0023] In a preferred embodiment of the present invention, the transporting is effected by receptor-mediated transcytosis or adsorptive-mediated transcytosis.

[0024] In a preferred embodiment of the present invention, the agent is for treatment of a neurological disease.

[0025] In a preferred embodiment of the present invention, the neurological disease is selected from the group consisting of a brain tumor, a brain metastasis, schizophrenia, epilepsy, Alzheimer's disease,

Parkinson's disease, Huntington's disease, stroke and blood-brain barrier related malfunctions.

[0026] In a preferred embodiment of the present invention, the blood-brain barrier related malfunction disease is obesity.

[0027] In a preferred embodiment of the present invention, the transporting results in delivery of the agent to the central nervous system (CNS) of an individual.

[0028] In a preferred embodiment of the present invention, the agent is releasable from the carrier after transport across the blood-brain barrier.

[0029] In a preferred embodiment of the present invention, the agent is released from the carrier after transport across the blood-brain barrier.

[0030] In a preferred embodiment of the present invention, there is provided a pharmaceutical composition for transporting an agent across a blood-brain barrier, the composition comprising a carrier according to an embodiment of the present invention in association with a pharmaceutically acceptable excipient.

[0031] In accordance with another embodiment of the present invention, there is provided a pharmaceutical composition for treating a neurological disease comprising a carrier according to an embodiment of the present invention in association with a pharmaceutically acceptable excipient.

[0032] In accordance with another embodiment of the present invention, there is provided a pharmaceutical composition for delivery of an agent to the CNS of an individual, the composition comprising a carrier according to an embodiment of the present invention in association with a pharmaceutically acceptable excipient.

[0033] In accordance with another embodiment of the present invention, there is provided a conjugate for transporting an agent across a blood-brain barrier, the conjugate comprising: (a) a carrier; and (b) an agent attached to the carrier, wherein the conjugate is able to cross the blood-brain barrier and thereby transport the agent across the blood-brain barrier.

[0034] In accordance with another embodiment of the present invention, there is provided a pharmaceutical composition for transporting an agent across a blood-brain barrier, the composition comprising a conjugate according to an embodiment of the present invention in association with a pharmaceutically acceptable excipient.

[0035] In accordance with an embodiment of the present invention, there is provided a pharmaceutical composition for treating a neurological disease, the composition comprising a conjugate according to an embodiment of the present invention in association with a pharmaceutically acceptable excipient.

[0036] In accordance with another embodiment of the present invention, there is provided a pharmaceutical composition for delivery of an agent to the CNS of an individual, the composition comprising a conjugate according to an embodiment of the present invention in association with a pharmaceutically acceptable excipient.

[0037] In accordance with another embodiment of the present invention, there is provided a use of a carrier for transporting an agent attached thereto across a blood-brain barrier in the manufacture of a medicament for transporting the agent across the blood-brain barrier.

[0038] In accordance with another embodiment of the present invention, there is provided a pharmaceutical composition for transporting an agent across a blood-brain barrier, the composition comprising a medicament

manufactured as defined in an embodiment of the present invention in association with a pharmaceutically acceptable excipient.

[0039] In accordance with another embodiment of the present invention, there is provided a use of a carrier for transporting an agent attached thereto across a blood-brain barrier in the manufacture of a medicament for treating a neurological disease in an individual.

[0040] In accordance with another embodiment of the present invention, there is provided a pharmaceutical composition for treating a neurological disease comprising a medicament manufactured as defined in an embodiment of the present invention in association with a pharmaceutically acceptable excipient.

[0041] In accordance with another embodiment of the present invention, there is provided a use of a carrier for transporting an agent attached thereto across a blood-brain barrier in the manufacture of a medicament for treating a central nervous system disorder in an individual.

[0042] In accordance with another embodiment of the present invention, there is provided a pharmaceutical composition for treating a central nervous system disorder, the composition comprising a medicament manufactured as defined in an embodiment of the present invention in association with a pharmaceutically acceptable excipient.

[0043] In accordance with another embodiment of the present invention there is provided a conjugate of formula R-L-M or a pharmaceutically acceptable salt thereof, wherein R is a carrier able to cross the blood-brain barrier after attachment to L-M and thereby transport M across the blood-brain barrier, L is a linker or a chemical bond and M is an agent selected from the group consisting of a drug, a medicine, a protein, a peptide, an enzyme, an antibiotic, an anti-cancer agent, a molecule active at the level

of the central nervous system, a radioimaging agent, an antibody, a cellular toxin, a detectable label and an anti-angiogenic compound.

[0044] In accordance with another embodiment of the present invention, there is provided a use of a conjugate according to an embodiment of the present invention for transporting an agent attached thereto across a blood-brain barrier.

[0045] In accordance with another embodiment of the present invention, there is provided a use of a conjugate according to an embodiment of the present invention for treating a neurological disease in an individual.

[0046] In accordance with another embodiment of the present invention, there is provided a use of a conjugate according to an embodiment of the present invention for treating a central nervous system disorder in an individual.

[0047] In accordance with another embodiment of the present invention, there is provided a method for transporting an agent across a blood-brain barrier, which comprises the step of administering to an individual a pharmaceutical composition according to an embodiment of the present invention.

[0048] In a preferred method of the present invention the pharmaceutical composition is administered to the individual intra-arterially, intra-nasally, intra-peritoneally, intravenously, intramuscularly, subcutaneously, transdermally or *per os*.

[0049] In accordance with another embodiment of the present invention, there is provided a method for treating a neurological disease in an individual comprising administering to the individual in need thereof a therapeutically effective amount of a pharmaceutical composition according to an embodiment of the present invention.

[0050] In accordance with another embodiment of the present invention, there is provided a method for treating a central nervous system disorder in an individual comprising administering to the individual in need thereof a therapeutically effective amount of a pharmaceutical composition according to an embodiment of the present invention.

[0051] For the purpose of the present invention the following terms are defined below.

[0052] The term "carrier" or "vector" is intended to mean a compound or molecule that is able to cross the blood-brain barrier and be attached to or conjugated to another compound or agent and thereby be able to transport the other compound or agent across the blood-brain barrier. For example, the carrier may bind to receptors present on brain endothelial cells and thereby be transported across the blood-brain barrier by transcytosis. Preferably the carrier is a protein or molecule for which very high levels of transendothelial transport are obtained without any effects on the blood-brain barrier integrity. The carrier may be, but is not limited to, a protein, a peptide, or a peptidomimetic and can be naturally occurring or produced by chemical synthesis or recombinant genetic technology (genetic engineering).

[0053] The term "carrier-agent conjugate" is intended to mean a conjugate of a carrier and another compound or agent. The conjugation can be chemical in nature, such as with a linker, or genetic in nature for example by recombinant genetic technology, such as in a fusion protein with for example green fluorescent protein, β -galactosidase or Histag protein.

[0054] The expression "small molecule drug" is intended to mean a drug having a molecular weight of 1000 g/mol or less.

[0055] The terms "treatment", "treating" and the like are intended to mean obtaining a desired pharmacologic and/or physiologic effect, e.g., inhibition of cancer cell growth, death of a cancer cell or amelioration of a neurological disease or condition. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment" as used herein covers any treatment of a disease in a mammal, particularly a human, and includes: (a) preventing a disease or condition (e.g., preventing cancer) from occurring in an individual who may be predisposed to the disease but has not yet been diagnosed as having it; (b) inhibiting a disease, (e.g., arresting its development); or (c) relieving a disease (e.g., reducing symptoms associated with a disease). "Treatment" as used herein covers any administration of a pharmaceutical agent or compound to an individual to treat, cure, alleviate, improve, diminish or inhibit a condition in the individual, including, without limitation, administering a carrier-agent conjugate to an individual.

[0056] The term "cancer" is intended to mean any cellular malignancy whose unique trait is the loss of normal controls which results in unregulated growth, lack of differentiation and ability to invade local tissues and metastasize. Cancer can develop in any tissue of any organ. More specifically, cancer is intended to include, without limitation, cancer of the brain.

[0057] The term "administering" and "administration" is intended to mean a mode of delivery including, without limitation, intra-arterially, intranasally, intra-peritoneally, intravenously, intramuscularly, sub-cutaneously, transdermally or *per os*. The preferred one being *per os*. A daily dosage can be divided into one, two or more doses in a suitable form to be administered at one, two or more times throughout a time period.

[0058] The term "therapeutically effective" is intended to mean an amount of a compound sufficient to substantially improve some symptom associated with a disease or a medical condition. For example, in the treatment of cancer or a mental condition or neurological or CNS disease, an agent or compound which decreases, prevents, delays, suppresses, or arrests any symptom of the disease or condition would be therapeutically effective. A therapeutically effective amount of an agent or compound is not required to cure a disease or condition but will provide a treatment for a disease or condition such that the onset of the disease or condition is delayed, hindered, or prevented, or the disease or condition symptoms are ameliorated, or the term of the disease or condition is changed or, for example, is less severe or recovery is accelerated in an individual.

[0059] The carrier and carrier-agent conjugates of the present invention may be used in combination with either conventional methods of treatment and/or therapy or may be used separately from conventional methods of treatment and/or therapy.

[0060] When the carrier-agent conjugates of this invention are administered in combination therapies with other agents, they may be administered sequentially or concurrently to an individual. Alternatively, pharmaceutical compositions according to the present invention may be comprised of a combination of a carrier-agent conjugate of the present invention in association with a pharmaceutically acceptable excipient, as described herein, and another therapeutic or prophylactic agent known in the art.

[0061] It will be understood that a specific "effective amount" for any particular individual will depend upon a variety of factors including the activity of the specific agent employed, the age, body weight, general health, sex, and/or diet of the individual, time of administration, route of

administration, rate of excretion, drug combination and the severity of the particular disease undergoing prevention or therapy.

[0062] Pharmaceutically acceptable acid addition salts may be prepared by methods known and used in the art.

[0063] As used herein, "pharmaceutically acceptable carrier" includes any and all solvents (such as phosphate buffered saline buffers, water, saline), dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

[0064] The term "functional derivative" is intended to mean a "chemical derivative", "fragment", or "variant" biologically active sequence or portion of a carrier or agent or carrier-agent conjugate or a salt thereof of the present invention. A carrier functional derivative is able to be attached to or conjugated to another compound or agent and cross the blood-brain barrier and thereby be able to transport the other compound or agent across the blood-brain barrier.

[0065] The term "chemical derivative" is intended to mean a carrier, an agent, or a carrier-agent conjugate of the present invention, which contains additional chemical moieties not a part of the carrier, agent or carrier-agent conjugate. Covalent modifications are included within the scope of this invention. A chemical derivative may be conveniently prepared by direct chemical synthesis, using methods well known in the art. Such modifications may be, for example, introduced into a protein or peptide carrier, agent or carrier-agent conjugate by reacting targeted amino acid residues with an organic derivatizing agent that is capable of reacting with

selected side chains or terminal residues. A carrier chemical derivative is able to cross the blood-brain barrier and be attached to or conjugated to another compound or agent and thereby be able to transport the other compound or agent across the blood-brain barrier. In a preferred embodiment, very high levels of transendothelial transport across the blood-brain barrier are obtained without any effects on the blood-brain barrier integrity.

[0066] The term "fragment" is intended to mean any piece or portion of a carrier, agent or carrier-agent conjugate. A fragment of a protein or peptide, for example, may be a subset of amino acids which makes up the sequence of the whole protein or peptide. A carrier fragment is able to be attached to or conjugated to another compound or agent and cross the blood-brain barrier and thereby be able to transport the other compound or agent across the blood-brain barrier.

[0067] The term "variant" is intended to mean to a carrier, agent or carrier-agent conjugate which is substantially similar to either the structure of a carrier, agent or carrier-agent conjugate, or any fragment thereof, of the present invention. A carrier variant is able to be attached to or conjugated to another compound or agent and cross the blood-brain barrier and thereby be able to transport the other compound or agent across the blood-brain barrier. Variant proteins, peptides, peptidomimetics and chemical structures of carriers of the present invention are contemplated.

[0068] The term "aprotinin fragment" is intended to mean a portion of aprotinin that can still transport a compound across the BBB. Such a fragment can comprise at least 12 amino acids, preferably at least 25 amino acids and more preferably at least 35 amino acids. Studies to determine the minimal sequence of aprotinin effective to interact with megalin have been performed by Hussain, M., Strickland, D. K., Bakillah, A., in *The mammalian low-density lipoprotein receptor family. Anno. Rev.*

Nutr. 1999, 19, 141-172. For example, the minimal sequence for interaction of Aprotinin with Megalin receptor was determined to be CRAKRNNFKSA (SEQ ID NO:1). Accordingly, fragments comprising this minimal sequence are meant to be included by this term.

[0069] The term "agent" is intended to mean without distinction a drug or a compound such as a therapeutic agent or compound, a marker, a tracer or an imaging compound.

[0070] The term "therapeutic agent" or "agent" is intended to mean an agent and/or medicine and/or drug used to treat the symptoms of a disease, physical or mental condition, injury or infection and includes, but is not limited to, antibiotics, anti-cancer agents, anti-angiogenic agents and molecules active at the level of the central nervous system Paclitaxel, for example, can be administered intravenously to treat brain cancer.

[0071] The term "patient" or "individual treated" is intended to mean any one who receives a certain medical treatment, and includes being subjected to the administration of a carrier-agent or compound conjugate for detecting, tracing, marking or imaging a condition, such as a tumor. Preferably, the patient or individual treated is a mammal and more preferably a human.

[0072] The term "condition" is intended to mean any situation causing pain, discomfort, sickness, disease or disability (mental or physical) to or in an individual, including neurological disease, injury, infection, or chronic or acute pain. Neurological diseases which can be treated with the present invention include, but are not limited to, brain tumors, brain metastases, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease and stroke.

BRIEF DESCRIPTION OF THE DRAWINGS

[0073] Fig. 1 is a plot showing the results of transcytosis experiments of aprotinin (●), p97 (◆), and ceruloplasmin (■) across bovine brain capillary endothelial cells (BBCECs);

[0074] Fig. 2 is a plot showing the results of transcytosis experiments of aprotinin (●) and transferrin (○) across bovine brain capillary endothelial cells (BBCECs);

[0075] Fig. 3 is a bar graph illustrating that aprotinin has a higher transcytosis capacity than transferrin in a blood-brain barrier model;

[0076] Fig. 4 is an SDS-PAGE analysis illustrating that aprotinin integrity is not affected by its transcytosis across BBCEC monolayers;

[0077] Fig. 5 is a plot of the clearance of [¹⁴C]-sucrose expressed as a function of time. The clearance of sucrose was measured in the presence and the absence of 250 nM aprotinin;

[0078] Fig. 6 is a graph showing the results of a sucrose permeability test of bovine brain capillary endothelial cells (BBCECs)

[0079] Fig. 7 is a plot of the clearance of [¹⁴C]-sucrose expressed as a function of time illustrating that aprotinin does not affect blood-brain barrier integrity. The clearance of sucrose was measured in the presence and the absence of 5 μM aprotinin;

[0080] Fig. 8 is a bar graph illustrating the accumulation of [¹²⁵I]-aprotinin in human and rat capillaries;

[0081] Fig. 9 is a plot illustrating a time-course of aprotinin uptake in human and rat capillaries

[0082] Fig. 10 is a bar graph illustrating that aprotinin-biotin conjugate and aprotinin have the same transcytosis capacity;

[0083] Fig. 11 is a bar graph illustrating that aprotinin and aprotinin-biotin conjugate transcytosis is temperature-dependent and conformational-dependent;

[0084] Figs. 12A and 12B are sets of plots illustrating the effect of temperature and heating on (A) aprotinin and (B) aprotinin-biotin conjugate transcytosis in BBCEC cells;

[0085] Fig. 13 is a bar graph illustrating the increase in streptavidin transcytosis in the presence of aprotinin-biotin conjugate;

[0086] Fig. 14 is a bar graph illustrating the inhibition of aprotinin transcytosis by the LRP antagonist, receptor-associated protein (RAP);

[0087] Fig. 15 is a bar graph illustrating aprotinin uptake in an *in situ* brain perfusion experiment;

[0088] Fig. 16 illustrates a synthetic-aprotinin sequence;

[0089] Fig. 17 illustrates a sequence alignment between aprotinin and three human proteins with a similar domain;

[0090] Fig. 18 is a bar graph illustrating *in situ* brain perfusion of transferrin, aprotinin and Angio-pep1;

[0091] Fig. 19 is a plot illustrating transcytosis of Angio-pep1 compared to that of aprotinin; and

[0092] Fig. 20 is a plot illustrating transcytosis of Angio-pep1 across the *in vitro* blood-brain barrier model.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

[0093] The present invention relates to a new vector or carrier to transport an agent, medicine or other molecule to the brain and/or central nervous system (CNS). This carrier permits the passage of the agent, medicine or other molecule which is attached or coupled (conjugated) to the carrier and which are unable by themselves to cross the blood-brain barrier, to be transported across the blood-brain barrier. The carrier-conjugate can be a carrier-therapeutic agent conjugate. Such conjugates can be in the form of a composition, such as a pharmaceutical composition, for treatment of a condition or disease. This invention is based on the discovery that aprotinin binds to and crosses the brain capillary endothelial wall in a very effective manner. Aprotinin is known in the art to be a basic polypeptide that effectively inhibits a variety of serine proteases, including trypsin, chymotrypsin, kallikrein and pepsin. The transendothelial transport of aprotinin is approximately 10-50 times higher than that of other proteins including transferrin or ceruloplasmin. This high rate of passage is not caused by the disruption of the integrity of the blood-brain barrier since the permeability coefficient for sucrose is not affected by aprotinin.

[0094] This approach is very versatile since it permits conjugation of small as well as large molecules having very diverse therapeutic targets.

[0095] In accordance with the present invention a method for transporting an agent across the blood-brain barrier comprises administering to an individual an agent that comprises an active ingredient or a pharmaceutical agent attached to a carrier, such as aprotinin, or a functional derivative thereof.

[0096] In accordance with the present invention, the compound can be administered intra-arterially, intra-nasally, intra-peritoneally, intravenously, intramuscularly, sub-cutaneously, transdermally or *per os* to the patient. The agent is preferably an anti-angiogenic compound. The agent can have

a maximum weight of 160,000 Daltons. Preferably, the agent is a marker or a drug such as a small molecule drug, a protein, a peptide or an enzyme. The drug preferably is adapted to treat a neurological disease or a central nervous system disorder of a patient. The drug can be a cytotoxic drug and the marker can be a detectable label such as a radioactive label, a green fluorescent protein, a histag protein or β -galactosidase. The agent is preferably delivered into the central nervous system of a patient.

[0097] According to still another preferred embodiment of the invention, the uses, methods, compounds, agents, drugs or medicaments of the invention do not alter the integrity of the blood-brain barrier of the patient.

[0098] According to a further preferred embodiment of the invention, aprotinin can be attached to an agent or a compound for transporting the agent or compound across the blood-brain barrier of a patient, the agent or compound being adapted to treat a neurological disease or to treat a central nervous system disorder.

[0099] The carrier or functional derivative thereof of the present invention or mixtures thereof may be linked to or labelled with a detectable label such as a radioimaging agent, such as those emitting radiation, for detection of a disease or condition, for example by the use of a radioimaging agent-antibody-carrier conjugate, wherein the antibody binds to a disease or condition-specific antigen. Other binding molecules besides antibodies and which are known and used in the art are also contemplated by the present invention. Alternatively, the carrier or functional derivative thereof of the present invention or mixtures thereof may be linked to a therapeutic agent, to treat a disease or condition, or may be linked to or labelled with mixtures thereof. Treatment is effected by administering a carrier-agent conjugate of the present invention to an individual under conditions which allow transport of the agent across the blood-brain barrier.

- 20 -

[00100] A therapeutic agent of the present invention can be a drug, a medicine, an agent emitting radiation, a cellular toxin (for example, a chemotherapeutic agent) and/or biologically active fragment thereof, and/or mixtures thereof to allow cell killing or it may be an agent to treat, cure, alleviate, improve, diminish or inhibit a disease or condition in an individual treated. A therapeutic agent can be a synthetic product or a product of fungal, bacterial or other microorganism, such as mycoplasma, viral etc., animal, such as reptile, or plant origin. A therapeutic agent and/or biologically active fragment thereof can be an enzymatically active agent and/or fragment thereof, or can act by inhibiting or blocking an important and/or essential cellular pathway or by competing with an important and/or essential naturally occurring cellular component.

[00101] Radioimaging agents emitting radiation (detectable radio-labels) for use in the present invention are exemplified by indium-111, technitium-99, or low dose iodine-131.

[00102] Detectable labels, or markers, for use in the present invention can be a radiolabel, a fluorescent label, a nuclear magnetic resonance active label, a luminescent label, a chromophore label, a positron emitting isotope for PET scanner, chemiluminescence label, or an enzymatic label. Fluorescent labels include, but are not limited to, green fluorescent protein (GFP), fluorescein, and rhodamine. Chemiluminescence labels include, but are not limited to, luciferase and β -galactosidase. Enzymatic labels include, but are not limited to peroxidase and phosphatase. A histag may also be a detectable label.

[00103] It is contemplated that an agent may be releasable from the carrier after transport across the blood-brain barrier, for example by enzymatic cleavage or breakage of a chemical bond between the carrier and the agent. The release agent would then function in its intended capacity in the absence of the carrier.

[00104] The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXPERIMENTAL SECTION

DETERMINATION OF A SUITABLE CARRIER

[00105] A reproducible blood-brain barrier *in vitro* model demonstrating *in vivo* characteristics has been used for screening assay and for mechanistic studies of drug transport to the brain. This efficient *in vitro* model of the blood-brain barrier was developed by the company CELLIAL™ Technologies was of prime importance to the reliable evaluation of the capacity of different carriers to reach the brain. The model consists of a co-culture of bovine brain capillary endothelial cells and rat glial cells. It presents ultrastructural features characteristic of brain endothelium including tight junctions, lack of fenestration, lack of transendothelial channels, low permeability for hydrophilic molecules and a high electrical resistance. Moreover, this model has shown a good correlation coefficient between *in vitro* and *in vivo* analysis of wide range of molecules tested. To date, all the data obtained show that this BBB model closely mimics the *in vivo* situation by reproducing some of the complexities of the cellular environment that exist *in vivo*, while retaining the experimental advantages associated with tissue culture. Thus, many studies have validated this cell co-culture as one of the most reproducible *in vitro* model of the BBB.

[00106] The *in vitro* model of BBB was established by using a co-culture of BBCECs and astrocytes. Prior to cell culture, plate inserts (Millicell-PC 3.0 μ M ; 30-mm diameter) were coated on the upper side with rat tail collagen. They were then set in six-well microplates containing the astrocytes and BBCECs were plated on the upper side of the filters in 2 mL of co-culture medium. This BBCEC medium was changed three times a week. Under these conditions, differentiated BBCECs formed a confluent

monolayer 7 days later. Experiments were performed between 5 and 7 days after confluence was reached. The permeability coefficient for sucrose was measured to verify the endothelial permeability.

[00107] Primary cultures of mixed astrocytes were prepared from newborn rat cerebral cortex (Dehouck M.P., Meresse S., Delorme P., Fruchart J.C., Cecchelli, R. An Easier, Reproducible, and Mass-Production Method to Study the Blood-Brain Barrier In Vitro. *J.Neurochem*, 54, 1798-1801, 1990). Briefly, after removing the meninges, the brain tissue was forced gently through a 82 μ m nylon sieve. Astrocytes were plated on six-well microplates at a concentration of 1.2×10^5 cells/mL in 2 mL of optimal culture medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum. The medium was changed twice a week.

[00108] Bovine brain capillary endothelial cells (BBCECs) were obtained from Cellial Technologies. The cells were cultured in the presence of DMEM medium supplemented with 10% (v/v) horse serum and 10% heat-inactivated calf serum, 2 mM of glutamine, 50 μ g/mL of gentamycin, and 1 ng/mL of basic fibroblast growth factor, added every other day.

[00109] In order to determine a suitable carrier for the present invention, tests have been performed using the *in vitro* model of the BBB. As illustrated in Fig. 1, transcytosis experiments of different proteins (aprotinin (●), p97 (♦) and ceruloplasmin (■)) across bovine brain capillary endothelial cells (BBCECs) were performed. Figs. 2 and 3 show the results of transcytosis experiments performed with aprotinin (●) and transferrin (○) and using the same method than the experiments of Fig. 1. One insert covered with BBCECs was set into a six-well microplate with 2 mL of Ringer-Hepes and was pre-incubated for 2 h at 37°C. [125 I]-aprotinin, [125 I]-p97, [125 I]-ceruloplasmin or [125 I]-transferrin (250 nM final concentration) was added to the upper side of the filter covered with cells. At various times, the insert was transferred to another well to avoid a

possible reendocytosis of [125 I]-proteins by the abluminal side of the BBCECs. At the end of experiment, [125 I]-proteins were assessed in 500 μ L of the lower chamber of well by TCA precipitation. The results indicate that aprotinin has a higher transcytosis capacity than transferrin, p97 or ceruloplasmin in a blood-brain barrier model.

[00110] Aprotinin, p97 and bovine holo-transferrin were iodinated with standard procedures using iodo-beads from SigmaTM. Bovine holo-transferrin was diluted in 0.1M phosphate buffer, pH 6.5 (PB). P97 obtained from Synapse Technologies in neutralized citrate at pH 7.0 was dialyzed against this PB. Two iodo-beads were used for each protein. These beads were washed twice with 3 mL of PB on a WhatmanTM filter and resuspended in 60 μ L of PB. 125 I (1 mCi) from Amersham-Pharmacia biotech was added to the bead suspension for 5 minutes at room temperature. The iodination for each protein was initiated by the addition of 100 μ g (80-100 μ L). After an incubation of 10 minutes at room temperature, the supernatants were applied on a desalting column prepacked with 5 mL of cross-linked dextran from Pierce and 125 I-proteins were eluted with 10 mL of PBS. Fractions of 0.5 mL were collected and the radioactivity in 5 μ L of each fraction was measured. Fractions corresponding to 125 I-proteins were pooled and dialyzed against Ringer-Hepes, pH 7.4. The efficiency of radiolabeling was between $0.6-1 \times 10^8$ cpm/100 μ g of protein.

[00111] From Figs. 1-3, it is clear that aprotinin has a transcytosis capacity which is quite higher than the other tested proteins. The data of Figs. 1-3 have been summarized in Table 1, wherein a comparison of the different proteins has been made.

Table 1

Comparison of ^{125}I -proteins (250 nM) transcytosis across BBCEC monolayers

| Proteins compared | Ratios (x-fold) |
|---------------------------|------------------------|
| aprotinin / p97 | 8.2 |
| aprotinin / ceruloplasmin | 44.0 |
| aprotinin / transferrin | 11.6 |

[00112] Table 2 summarizes another experiment, wherein a comparison of additional different proteins has been made.

Table 2

Efficiency of aprotinin to cross the blood-brain barrier

| Proteins compared | Transcytosis (pmol/h/cm²) | Ratios Aprotinin/Protein |
|--------------------------|---|-------------------------------------|
| Aprotinin | 2.7 | 1 |
| Melanotransferrin (p97) | 0.28 | 10 |
| Transferrin | 0.14 | 19 |
| Lactoferrin | 0.05 | 50 |
| Streptavidin | 0.09 | 30 |

[00113] In view of Tables 1 and 2, it can be seen that for aprotinin, a superior transendothelial transport was obtained in comparison with the other tested proteins and that the high transcytosis of aprotinin is from about 10 to 50-fold higher than these other proteins.

**APROTININ INTEGRITY IS NOT AFFECTED BY ITS TRANSCYTOSIS
ACROSS BBCEC MONOLAYERS**

[00114] [¹²⁵I]-protein (0.5-1.5 μ Ci/assay) at a final concentration of 250nM was added to the upper side of filters with or without BBCEC cells placed in 6-well plates. At each time point, filters were put in the next well of the 6-well plates. At the end of the experiment, aliquots were taken in each well and submitted to SDS-PAGE. Gels were then submitted to detection by autoradiography. The results, presented in Fig. 4, indicate that aprotinin integrity is not affected by its transcytosis across BBCEC monolayers.

**APROTININ DOES NOT AFFECT THE BLOOD-BRAIN BARRIER
INTEGRITY**

[00115] A further test was performed to determine the effect of aprotinin at 250 nM on the BBB integrity by measuring [¹⁴C] sucrose permeability in the BBB model on BBCEC monolayers grown on filters in the presence of astrocytes. To achieve this test, brain endothelial cell monolayers grown on inserts were transferred to 6-well plates containing 2 mL of Ringer-Hepes per well (basolateral compartment) for two hours at 37 °C. Ringer-Hepes solution was composed of 150 mM NaCl, 5.2 mM KCl, 2.2 mM CaCl₂, 0.2 mM MgCl₂, 6 mM NaHCO₃, 5 mM Hepes, 2.8 mM Hepes, pH 7.4. In each apical chamber, the culture medium was replaced by 1 mL Ringer-Hepes containing the labeled [¹⁴C]-sucrose. At different times, inserts were placed into another well. [¹⁴C] sucrose passage was

measured at 37°C, on filters without cells (\square) or with filters coated with BBCEC cells in the absence (Δ) or presence (\circ) of 5 μ M aprotinin (Fig. 6). The results were plotted as the sucrose clearance (μ l) as a function of time (min). The sucrose permeability coefficient was then determined. The permeability coefficient (Pe) was calculated as:

$$1) \text{ Clearance } (\mu\text{l}) = \frac{[C]A \times VA}{[C]L}$$

wherein: $[C]A$ = Abluminal tracer concentration
 VA = Volume of abluminal chamber
 $[C]L$ = Luminal tracer concentration

$$2) 1/Pe = (1/PSt - 1/PSf) / \text{filter area } (4.2 \text{ cm}^2)$$

[00116] At the end of the experiments, amounts of the radiotracers in the basolateral compartment were measured in a liquid scintillation counter. The permeability coefficient (Pe) for sucrose was calculated as previously described (Dehouck, M.P., Jolliet-Riant, P., Brée, F., Fruchart, J.C., Cecchelli, R., Tillement, J.P., *J. Neurochem.* 58:1790-1797, 1992) using filters coated or non-coated with EC. The results of two experiments were plotted separately in terms of the clearance of [14 C]-sucrose (μ L) as a function of time (min) (Figs. 5 and 6). In Figs. 5 and 6, PSt represents the permeability x surface area of a filter of the coculture and PSf represents the permeability of a filter coated with collagen and astrocytes plated on the bottom side of the filter B. The permeability coefficient (Pe) was calculated and it was demonstrated that the integrity of the BBB is not affected by aprotinin (see Fig. 6 for Pe calculated from Fig. 5, and Table 3 for Pe calculated from Fig. 7).

Table 3

Permeability coefficients of aprotinin demonstrate that aprotinin does not affect the integrity of the blood-brain barrier

| Pe sucrose (10^{-3} cm/min) | |
|--------------------------------|-----------------|
| - Aprotinin | 0.46 ± 0.09 |
| + Aprotinin | 0.32 ± 0.04 |

**ACCUMULATION OF [125 I]-APROTININ IN HUMAN AND RAT
CAPILLARIES**

[00117] Accumulation was measured at 37°C for 1 hour. Incubation medium contained aprotinin at a final 100 nM concentration in Ringer/Hepes solution. Accumulation was stopped by addition of ice-cold stop-solution and filtration in vacuum through a 0.45 μ M filter. Nonspecific binding of aprotinin to the capillaries surface was evaluated by the addition of the ice-cold solution before adding the incubation medium. This value was subtracted from accumulation value to obtain the real accumulation value. The results of this experiment are shown in Fig. 8.

**TIME-COURSE OF APROTININ UPTAKE IN HUMAN AND RAT
CAPILLARIES**

[00118] Aprotinin uptake was measured at 37°C for variable time. Incubation medium contained aprotinin at a final 100 nM concentration in Ringer/Hepes solution. At each time point, accumulation was stopped by addition of ice-cold stop-solution and filtration in vacuum through a 0.45 μ M filter. At each time point, nonspecific binding of aprotinin to the capillaries surface was evaluated by the addition of the ice-cold solution before adding the incubation medium. The results of this experiment are shown in Fig. 9.

APROTININ-BIOTIN CONJUGATE: BIOTINYLATION PROCEDURE

[00119] Water soluble biotin analog Sulfo-NHS-LC-LC-Biotin (Pierce) was used for conjugation. This analog reacts with primary amines in the absence of organic solvent and at neutral pH. A 12-fold molar excess of biotin analog was added to a 10 mg/ml aprotinin solution. Biotin analog and aprotinin mix was incubated for 2 hours at 4°C. To remove unreacted biotin reagent, a dialysis was performed overnight in a slide-a-lyzer dialysis cassette (Pierce) with a 3500 Da cut-off. Determination of biotin incorporation was then performed with the dye HABA (2-(4'-hydroxyazobenzene)-benzoic acid) that binds to avidin yielding an absorption at 500 nm. This binding can be displaced with free biotin or with a biotinylated protein, allowing quantitation of biotin incorporation. The ratio obtained for this conjugation was three biotin for each aprotinin.

APROTININ-BIOTIN CONJUGATE AND APROTININ HAVE THE SAME TRANSCYTOSIS CAPACITY

[00120] Transcytosis of [125 I]-aprotinin and [125 I]-aprotinin-biotin was evaluated at 37°C. [125 I]-protein (0.5-1.5 μ Ci/assay) at a final concentration of 250nM was added to the upper side of the cell-covered filter for transcytosis measurement. At the end of the experiment, [125 I]-protein cellular transcytosis was determined directly by TCA precipitation. The results of this experiment are shown in Fig. 10.

**APROTININ AND APROTININ-BIOTIN CONJUGATE TRANSCYTOSIS IS
TEMPERATURE-DEPENDENT AND CONFORMATIONAL-DEPENDENT**

[00121] Accumulation of [125 I]-aprotinin and [125 I]-aprotinin-biotin was evaluated at 37°C and 4°C, or at 37°C after proteins had been boiled for 10 min at 100°C. [125 I]-protein (0.5-1.5 μ Ci/assay) at a final concentration of 250nM was added to the upper side of the cell-covered filter for transcytosis measurement. At the end of the experiment, cell-covered filters were cut and [125 I]-protein cellular accumulation was determined directly by TCA precipitation. The results of this experiment are shown in Fig. 11.

**EFFECT OF TEMPERATURE AND HEATING ON APROTININ AND
APROTININ-BIOTIN CONJUGATE TRANSCYTOSIS IN BBCEC CELLS**

[00122] Transcytosis of [125 I]-aprotinin (Fig. 12A) and [125 I]-aprotinin-biotin (Fig. 12B) was evaluated at 37°C and 4°C, or at 37°C after proteins had been boiled for 10 min at 100°C. [125 I]-protein (0.5-1.5 μ Ci/assay) at a final concentration of 250nM was added to the upper side of the cell-covered filter for transcytosis measurement. At each time point filter was moved to the next well of the 6-well plate. At the end of the experiment, [125 I]-protein was assessed in the lower compartment of each well by TCA precipitation.

**INCREASE IN STREPTAVIDIN TRANSCYTOSIS IN THE PRESENCE OF
APROTININ-BIOTIN CONJUGATE**

[00123] Transcytosis of [125 I]-streptavidin was evaluated alone or in the presence of aprotinin-biotin conjugate. [125 I]-protein (0.5-1.5 μ Ci/assay) at a

- 30 -

final concentration of 250nM was added to the upper side of the cell-covered filter for transcytosis measurement. At each time point filter was moved to the next well of the 6-well plate. At the end of the experiment, [125 I]-protein was assessed in the lower compartment of each well by TCA precipitation. The results of this experiment are shown in Fig. 13.

INHIBITION OF APROTININ TRANSCYTOSIS BY THE LRP ANTAGONIST, RECEPTOR-ASSOCIATED PROTEIN (RAP)

[00124] Protein transcytosis was evaluated at 37°C. [125 I]-aprotinin (0.5-1.5 μ Ci/assay) at a final concentration of 250nM was added to the upper side of the cell-covered filter with or without rap. At the end of the experiment, [125 I]-aprotinin was assessed in the lower compartment of each well by TCA precipitation. The results of this experiment are shown in Fig. 14.

APROTININ UPTAKE: *IN SITU* MOUSE BRAIN PERFUSION

Surgical Procedure

[00125] The uptake of [125 I]-aprotinin to the luminal side of mouse brain capillaries was measured using the in situ brain perfusion method adapted in our laboratory for the study of drug uptake in the mouse brain (Dagenais et al., 2000, J. Cereb. Blood Flow Metab. 20(2):381-386). Briefly, the right common carotid of ketamine/xylazine (140/8 mg/kg i.p.) anesthetized mice was exposed and ligated at the level of the bifurcation of the common carotid, rostral to the occipital artery. The common carotid was then catheterized rostrally with polyethylene tubing (0.30 mm i.d. \times 0.70 mm o.d.) filled with heparin (25 U/ml) and mounted on a 26-gauge needle. The syringe containing the perfusion fluid (10 nM of [125 I]-aprotinin in

Krebs/bicarbonate buffer at a pH 7.4 gassed with 95% O₂ and 5% CO₂) was placed in an infusion pump (Harvard pump PHD 2000; Harvard Apparatus) and connected to the catheter. Immediately before the perfusion, the heart was stopped by severing the ventricles to eliminate contralateral blood flow contribution. The brain was perfused for 10 min at a flow rate of 2.5ml/min. After 10 min of perfusion, the brain was further perfused for 30 s with Ringer/HEPES solution (150 mM NaCl, 5.2 mM KCl, 2.2 mM CaCl₂, 0.2 mM MgCl₂, 6 mM NaHCO₃, 5 mM HEPES, 2.8 mM glucose, pH 7.4), to wash the excess of [¹²⁵I]-aprotinin. Mice were then decapitated to terminate perfusion and the right hemisphere was isolated on ice before being subjected to capillary depletion (Triguero et al., 1990, J Neurochem. 54(6):1882-8). Aliquots of homogenates, supernatants, pellets and perfusates were taken to measure their contents in [¹²⁵I]-aprotinin by TCA precipitation and to evaluate the apparent volume of distribution.

Determination of BBB transport constants

[00126] Briefly, calculations were carried out as previously described by Smith (1996, Pharm. Biotechnol. 8:285-307). Aprotinin uptake was expressed as the volume of distribution (V_d) from the following equation:

$$V_d = Q^*_{br} / C^*_{pf}$$

where Q^{*}_{br} is the calculated quantity of [¹²⁵I]-aprotinin per gram of right brain hemisphere and C^{*}_{pf} is the labeled tracer concentration measured in the perfusate.

[00127] The results of this experiment, shown in Fig. 15, indicate that there is higher brain uptake for aprotinin than transferrin and that conjugation with biotin does not modify brain uptake of aprotinin.

[00128] In view of the results obtained for the above-mentioned tests, aprotinin is a promising carrier for transporting an agent or compound across the BBB since it has a higher transcytosis across BBCEC monolayers than that of other proteins and it does not alter the integrity of the blood-brain barrier. In addition, aprotinin is not degraded during transcytosis nor does conjugation of aprotinin to biotin affect its transcytosis. Moreover, aprotinin is a versatile and flexible carrier since many molecules such as small drug molecules, proteins, peptides and enzymes may be easily attached to aprotinin proteins for promoting their passage across the BBB. These molecules can conceivably be attached to aprotinin via a linker.

[00129] It has also been determined that the brain distribution volume of aprotinin is higher than that of transferrin. It has further been determined that transcytosis is temperature sensible and conformation dependent, implying that a LDL-R family receptor, probably LRP is involved in aprotinin transcytosis.

[00130] Thus, aprotinin is an effective and efficient carrier to deliver an agent into the brain through the blood-brain barrier.

DESIGN OF A PEPTIDE AS A DRUG VECTOR FOR THE BRAIN

[00131] A sequence comparison was made on the N-terminal sequence of aprotinin (MRPDFCLEPPYTGPCVARIIR) (Fig. 16) (SEQ ID NO:2) using the BLAST™ program on the National Center for Biotechnology Information (NCBI) website. This sequence comparison resulted in four sequences being identified. None of these identified sequences corresponded to a human protein.

[00132] The C-terminal sequence of aprotinin (GLCQTFVYGGCRAKRNNFKSAE) (Fig. 16) (SEQ ID NO:3) was also

compared on the NCBI website. This sequence comparison resulted in 27 sequences being identified with some corresponding to human proteins. The proteins with the highest score were then aligned with the sequence of aprotinin (Fig. 17). From this alignment, the following Angio-pep1 peptide was generated: TFFYGGCRGKRNNFKTEEY (net charge +2) (SEQ ID NO:4).

IN SITU BRAIN PERFUSION OF TRANSFERRIN, APROTININ AND ANGIO-PEP1

[00133] The brain apparent volume of distribution was measured for [¹²⁵I]-transferrin, [¹²⁵I]-aprotinin and [¹²⁵I]-Angio-pep1. Mice brains were perfused for 10 min. Brain capillary depletion was performed to assess the apparent volume of distribution in the brain parenchyma. The results of this experiment are shown in Fig. 18.

TRANSCYTOSIS OF ANGIO-PEP1 COMPARED TO THAT OF APROTININ

[00134] Transcytosis of Angio-pep1 was compared to that of aprotinin. Transport of [¹²⁵I]-Angio-pep1 and [¹²⁵I]-aprotinin from the apical-to-basolateral side of endothelial cells monolayers was measured as described above. The final concentration used for angiopep1 and aprotinin for this experiment was 2.5 µM. The results of this experiment are shown in Fig. 19.

**TRANSCYTOSIS OF ANGIO-PEP1 ACROSS THE *IN VITRO* BLOOD-
BRAIN BARRIER MODEL**

[00135] The transport of Angio-pep1 from the apical-to-basolateral side of inserts covered with or without endothelial cell monolayers was measured. The results are expressed as the clearance of Angio-pep1 as a function of time. The slopes correspond to the permeability of the peptide through the filter alone (Psf) and to the total permeability of the endothelial cell monolayers (Pst). The permeability coefficient (Pe) for Angio-pep1 was 1.2×10^{-3} cm/min. The results of this experiment are shown in Fig. 20.

[00136] The permeability coefficients for Angio-pep1, aprotinin, leptin and transferrin were determined using the *in vitro* blood-brain barrier model. The permeability coefficient (Pe) was calculated as described above. The comparison of the permeability coefficients is shown in Table 4.

TABLE 4

Permeability coefficients for Angio-pep1, aprotinin, leptin and transferrin

| Proteins | Permeability coefficient (Pe) ($\times 10^{-3}$ cm/min) | Ratios |
|-------------|--|--------|
| Angio-pep1 | 1.2 | 1 |
| Aprotinin | 0.16 | 7.5 |
| Leptin | 0.055 | 21 |
| Transferrin | 0.0057 | 210 |

[00137] The above experiments indicate that brain penetration for Angio-pep1 is higher than that of aprotinin and transferrin. The experiments also indicate that transcytosis of Angio-pep1 measured using the *in vitro* blood-brain barrier model is higher than that of other proteins including aprotinin, leptin and transferrin.

[00138] While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A carrier for transporting an agent attached thereto across a blood-brain barrier, wherein said carrier is able to cross the blood-brain barrier after attachment to said agent and thereby transport said agent across the blood-brain barrier.
2. The carrier according to claim 1, wherein said transporting does not affect blood-brain barrier integrity.
3. The carrier according to claim 1, wherein said carrier is selected from the group consisting of aprotinin, a functional derivative of aprotinin, Angio-pep1 and a functional derivative of Angio-pep1.
4. The carrier according to claim 1, wherein said agent is selected from the group consisting of a drug, a medicine, a protein, a peptide, an enzyme, an antibiotic, an anti-cancer agent, a molecule active at the level of the central nervous system, a radioimaging agent, an antibody, a cellular toxin, a detectable label and an anti-angiogenic compound.
5. The carrier according to claim 4, wherein said anti-cancer agent is Paclitaxel.
6. The carrier according to claim 4, wherein said detectable label is selected from the group consisting of a radioactive label, a green fluorescent protein, a histag protein and β -galactosidase.
7. The carrier according to claim 1, wherein said agent has a maximum molecular weight of 160,000 Daltons.

- 37 -

8. The carrier according to claim 1, wherein said transporting is effected by receptor-mediated transcytosis or adsorptive-mediated transcytosis.
9. The carrier according to claim 1, wherein said agent is for treatment of a neurological disease.
10. The carrier according to claim 9, wherein said neurological disease is selected from the group consisting of a brain tumor, a brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and blood-brain barrier related malfunction disease.
11. The carrier according to claim 10, wherein said blood-brain barrier related malfunction disease is obesity.
12. The carrier according to claim 1, wherein said transporting results in delivery of said agent to the central nervous system (CNS) of an individual.
13. The carrier according to claim 1, wherein said agent is releasable from said carrier after transport across the blood-brain barrier.
14. The carrier according to claim 1, wherein said agent is released from said carrier after transport across the blood-brain barrier.
15. A pharmaceutical composition for transporting an agent across a blood-brain barrier, said composition comprising a carrier according to any one of claims 1 to 14 in association with a pharmaceutically acceptable excipient.

16. A pharmaceutical composition for treating a neurological disease, said composition comprising a carrier according to any one of claims 1 to 14 in association with a pharmaceutically acceptable excipient.
17. A pharmaceutical composition for delivery of an agent to the CNS of an individual, said composition comprising a carrier according to any one of claims 1 to 14 in association with a pharmaceutically acceptable excipient.
18. A conjugate for transporting an agent across a blood-brain barrier, said conjugate comprising: (a) a carrier; and (b) an agent attached to said carrier, wherein said conjugate is able to cross said blood-brain barrier and thereby transport said agent across said blood-brain barrier.
19. The conjugate according to claim 18, wherein said transporting does not affect blood-brain barrier integrity.
20. The conjugate according to claim 18, wherein said carrier is selected from the group consisting of aprotinin, a functional derivative of aprotinin, Angio-pep1 and a functional derivative of Angio-pep1.
21. The conjugate according to claim 18, wherein said agent is selected from the group consisting of a drug, a medicine, a protein, a peptide, an enzyme, an antibiotic, an anti-cancer agent, a molecule active at the level of the central nervous system, a radioimaging agent, an antibody, a cellular toxin, a detectable label and an anti-angiogenic compound.

22. The conjugate according to claim 21, wherein said anti-cancer agent is Paclitaxel.
23. The conjugate according to claim 21, wherein said detectable label is selected from the group consisting of a radioactive label, a green fluorescent protein, a histag protein and β -galactosidase.
24. The conjugate according to claim 18, wherein said agent has a maximum molecular weight of 160,000 Daltons.
25. The conjugate according to claim 18, wherein said transporting is effected by receptor-mediated transcytosis or adsorptive-mediated transcytosis.
26. The conjugate according to claim 18, for use in treating a neurological disease.
27. The conjugate according to claim 26, wherein said neurological disease is selected from the group consisting of a brain tumor, a brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and blood-brain barrier related malfunction disease.
28. The conjugate according to claim 27, wherein said blood-brain barrier related malfunction disease is obesity.
29. The conjugate according to claim 18, wherein said transporting results in delivery of said agent to the central nervous system (CNS) of an individual.

- 40 -

30. The conjugate according to claim 18, wherein said agent is releasable from said carrier after transport across the blood-brain barrier.
31. The conjugate according to claim 18, wherein said agent is released from said carrier after transport across the blood-brain barrier.
32. A pharmaceutical composition for transporting an agent across a blood-brain barrier, said composition comprising a conjugate according to any one of claims 18 to 31 in association with a pharmaceutically acceptable excipient.
33. A pharmaceutical composition for treating a neurological disease, said composition comprising a conjugate according to any one of claims 18 to 31 in association with a pharmaceutically acceptable excipient.
34. A pharmaceutical composition for delivery of an agent to the CNS of an individual, said composition comprising a conjugate according to any one of claims 18 to 31 in association with a pharmaceutically acceptable excipient.
35. Use of a carrier for transporting an agent attached thereto across a blood-brain barrier in the manufacture of a medicament for transporting said agent across said blood-brain barrier.
36. The use according to claim 35, wherein said transporting does not affect blood-brain barrier integrity.

37. The use according to claim 35, wherein said carrier is selected from the group consisting of aprotinin, a functional derivative of aprotinin, Angio-pep1 and a functional derivative of Angio-pep1.
38. The use according to claim 35, wherein said agent is selected from the group consisting of a drug, a medicine, a protein, a peptide, an enzyme, an antibiotic, an anti-cancer agent, a molecule active at the level of the central nervous system, a radioimaging agent, an antibody, a cellular toxin, a detectable label and an anti-angiogenic compound.
39. The use according to claim 38, wherein said anti-cancer agent is Paclitaxel.
40. The use according to claim 38, wherein said detectable label is selected from the group consisting of a radioactive label, a green fluorescent protein, a histag protein and β -galactosidase.
41. The use according to claim 35, wherein said agent has a maximum molecular weight of 160,000 Daltons.
42. The use according to claim 35, wherein said transporting is effected by receptor-mediated transcytosis or adsorptive-mediated transcytosis.
43. The use according to claim 35, wherein said carrier is for use in the treatment of a neurological disease.
44. The use according to claim 43, wherein said neurological disease is selected from the group consisting of a brain tumor, a brain metastasis, schizophrenia, epilepsy, Alzheimer's disease,

- 42 -

Parkinson's disease, Huntington's disease, stroke and blood-brain barrier related malfunction disease.

45. The use according to claim 44, wherein said blood-brain barrier related malfunction disease is obesity.
46. The use according to claim 35, wherein said transporting results in delivery of said agent to the central nervous system (CNS) of an individual.
47. The use according to claim 35, wherein said agent is releasable from said carrier after transport across the blood-brain barrier.
48. The use according to claim 35, wherein said agent is released from said carrier after transport across the blood-brain barrier.
49. A pharmaceutical composition for transporting an agent across a blood-brain barrier, said composition comprising a medicament as defined in any one of claims 35 to 48 in association with a pharmaceutically acceptable excipient.
50. Use of a carrier for transporting an agent attached thereto across a blood-brain barrier in the manufacture of a medicament for treating a neurological disease in an individual.
51. The use according to claim 50, wherein said transporting does not affect blood-brain barrier integrity.
52. The use according to claim 50, wherein said carrier is selected from the group consisting of aprotinin, a functional derivative of aprotinin, Angio-pep1 and a functional derivative of Angio-pep1.

53. The use according to claim 50, wherein said agent is selected from the group consisting of a drug, a medicine, a protein, a peptide, an enzyme, an antibiotic, an anti-cancer agent, a molecule active at the level of the central nervous system, a radioimaging agent, an antibody, a cellular toxin, a detectable label and an anti-angiogenic compound.
54. The use according to claim 53, wherein said anti-cancer agent is Paclitaxel.
55. The use according to claim 53, wherein said detectable label is selected from the group consisting of a radioactive label, a green fluorescent protein, a histag protein and β -galactosidase.
56. The use according to claim 50, wherein said agent has a maximum molecular weight of 160,000 Daltons.
57. The use according to claim 50, wherein said transporting is effected by receptor-mediated transcytosis or adsorptive-mediated transcytosis.
58. The use according to claim 50, wherein said neurological disease is selected from the group consisting of a brain tumor, a brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and blood-brain barrier related malfunction disease.
59. The use according to claim 58, wherein said blood-brain barrier related malfunction disease is obesity.

- 44 -

60. The use according to claim 50, wherein said transporting results in delivery of said agent to the central nervous system (CNS) of an individual.
61. The use according to claim 50, wherein said agent is releasable from said carrier after transport across the blood-brain barrier.
62. The use according to claim 50, wherein said agent is released from said carrier after transport across the blood-brain barrier.
63. A pharmaceutical composition for treating a neurological disease, said composition comprising a medicament as defined in any one of claims 50 to 62 in association with a pharmaceutically acceptable carrier.
64. Use of a carrier for transporting an agent attached thereto across a blood-brain barrier in the manufacture of a medicament for treating a central nervous system disorder in an individual.
65. The use according to claim 64, wherein said transporting does not affect blood-brain barrier integrity.
66. The use according to claim 64, wherein said carrier is selected from the group consisting of aprotinin, a functional derivative of aprotinin, Angio-pep1 and a functional derivative of Angio-pep1.
67. The use according to claim 64, wherein said agent is selected from the group consisting of a drug, a medicine, a protein, a peptide, an enzyme, an antibiotic, an anti-cancer agent, a molecule active at the level of the central nervous system, a radioimaging agent, an antibody, a cellular toxin, a detectable label and an anti-angiogenic compound.

68. The use according to claim 67, wherein said anti-cancer agent is Paclitaxel.
69. The use according to claim 67, wherein said detectable label is selected from the group consisting of a radioactive label, a green fluorescent protein, a histag protein and β -galactosidase.
70. The use according to claim 64, wherein said agent has a maximum molecular weight of 160,000 Daltons.
71. The use according to claim 64, wherein said transporting is effected by receptor-mediated transcytosis or adsorptive-mediated transcytosis.
72. The use according to claim 64, wherein said transporting results in delivery of said agent to the central nervous system (CNS) of an individual.
73. The use according to claim 64, wherein said agent is releasable from said carrier after transport across the blood-brain barrier.
74. The use according to claim 64, wherein said agent is released from said carrier after transport across the blood-brain barrier.
75. A pharmaceutical composition for treating a central nervous system disorder, said composition comprising a medicament as defined in any one of claims 64 to 74 in association with a pharmaceutically acceptable excipient.
76. Conjugates of formula R-L-M or a pharmaceutically acceptable salt thereof, for transporting M across a blood-brain barrier wherein R is a carrier able to cross said blood-brain barrier after attachment to

- 46 -

L-M and thereby transport M across said blood-brain barrier, L is a linker or a chemical bond and M is an agent is selected from the group consisting of a drug, a medicine, a protein, a peptide, an enzyme, an antibiotic, an anti-cancer agent, a molecule active at the level of the central nervous system, a radioimaging agent, an antibody, a cellular toxin, a detectable label and an anti-angiogenic compound.

77. The conjugate according to claim 76, wherein said transporting does not affect blood-brain barrier integrity.
78. The conjugate according to claim 76, wherein said carrier is selected from the group consisting of aprotinin, a functional derivative of aprotinin, Angio-pep1 and a functional derivative of Angio-pep1.
79. The conjugate according to claim 76, wherein said detectable label is selected from the group consisting of a radioactive label, a green fluorescent protein, a histag protein and β -galactosidase.
80. The conjugate according to claim 76, wherein said agent has a maximum molecular weight of 160,000 Daltons.
81. The conjugate according to claim 76, wherein said transporting is effected by receptor-mediated transcytosis or adsorptive-mediated transcytosis.
82. The conjugate according to claim 76, wherein M is an agent useful for treating a neurological disease.
83. The conjugate according to claim 82, wherein said neurological disease is selected from the group consisting of a brain tumor, a

- 47 -

brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and blood-brain barrier related malfunction disease.

84. The use according to claim 83, wherein said blood-brain barrier related malfunction disease is obesity.
85. The conjugate according to claim 76, wherein said transporting results in delivery of said agent to the central nervous system (CNS) of an individual.
86. The conjugate according to claim 76, wherein said agent is releasable from said carrier after transport across the blood-brain barrier.
87. The conjugate according to claim 76, wherein said agent is released from said carrier after transport across the blood-brain barrier.
88. A pharmaceutical composition for transporting an agent across a blood-brain barrier, said composition comprising a conjugate according to any one of claims 76 to 87 in association with a pharmaceutically acceptable excipient.
89. A pharmaceutical composition for treating a neurological disease, said composition comprising a conjugate according to any one of claims 76 to 87 in association with a pharmaceutically acceptable excipient.
90. A pharmaceutical composition for delivery of an agent to the CNS of an individual, said composition comprising a conjugate according

to any one of claims 76 to 87 in association with a pharmaceutically acceptable excipient.

91. Use of a conjugate according to any one of claims 18 to 31 and 76 to 87 for transporting an agent attached thereto across a blood-brain barrier.
92. Use of a conjugate according to any one of claims 18 to 31 and 76 to 87 for treating a neurological disease in an individual.
93. Use of a conjugate according to any one of claims 18 to 31 and 76 to 87 for treating a central nervous system disorder in an individual.
94. A method for transporting an agent across a blood-brain barrier, which comprises the step of administering to an individual a pharmaceutical composition according to any one of claims 15, 32, 49 and 88.
95. The method of claim 94, wherein said pharmaceutical composition is administered to said individual intra-arterially, intra-nasally, intra-peritoneally, intravenously, intramuscularly, sub-cutaneously, transdermally or *per os*.
96. The method of claim 94, wherein said pharmaceutical composition is administered to said individual *per os*.
97. A method for treating a neurological disease in an individual comprising administering to said individual in need thereof a therapeutically effective amount of a pharmaceutical composition according to any one of claims 16, 33, 63 and 89.

98. The method of claim 97, wherein said pharmaceutical composition is administered to said individual intra-arterially, intra-nasally, intra-peritoneally, intravenously, intramuscularly, sub-cutaneously, transdermally or *per os*.
99. The method of claim 97, wherein said pharmaceutical composition is administered to said individual *per os*.
100. A method for treating a central nervous system disorder in an individual comprising administering to said individual in need thereof a therapeutically effective amount of a pharmaceutical composition according to any one of claims 17, 34, 75 and 90.
101. The method of claim 100, wherein said pharmaceutical composition is administered to said individual intra-arterially, intra-nasally, intra-peritoneally, intravenously, intramuscularly, sub-cutaneously, transdermally or *per os*.
102. The method of claim 100, wherein said pharmaceutical composition is administered to said individual *per os*.

1/20

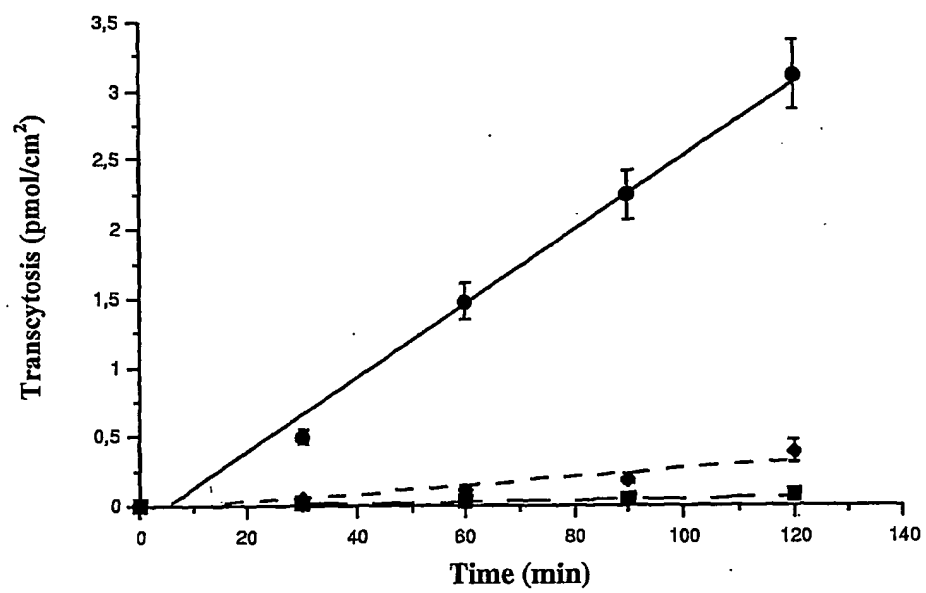


FIG. 1

2/20

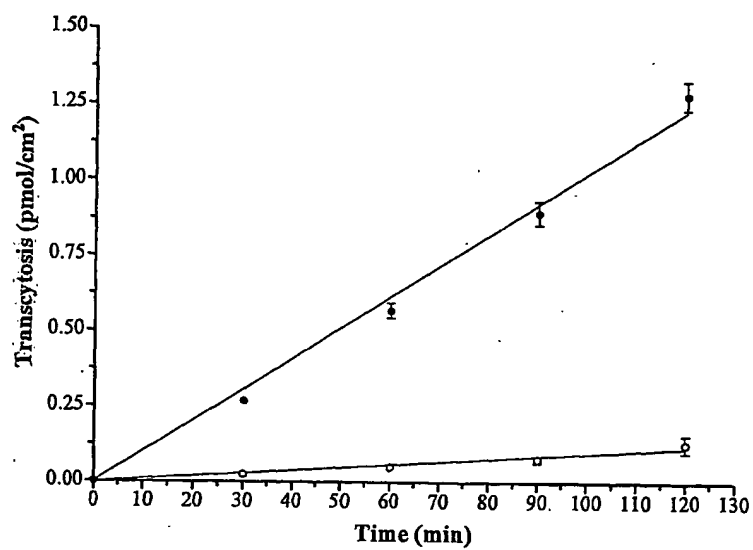


FIG. 2

3/20

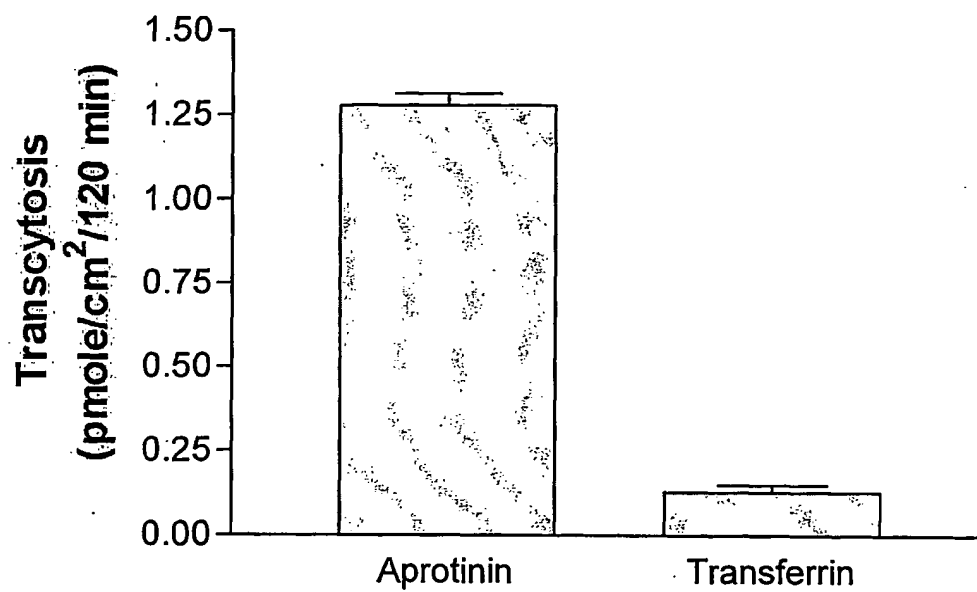


FIG. 3

4/20

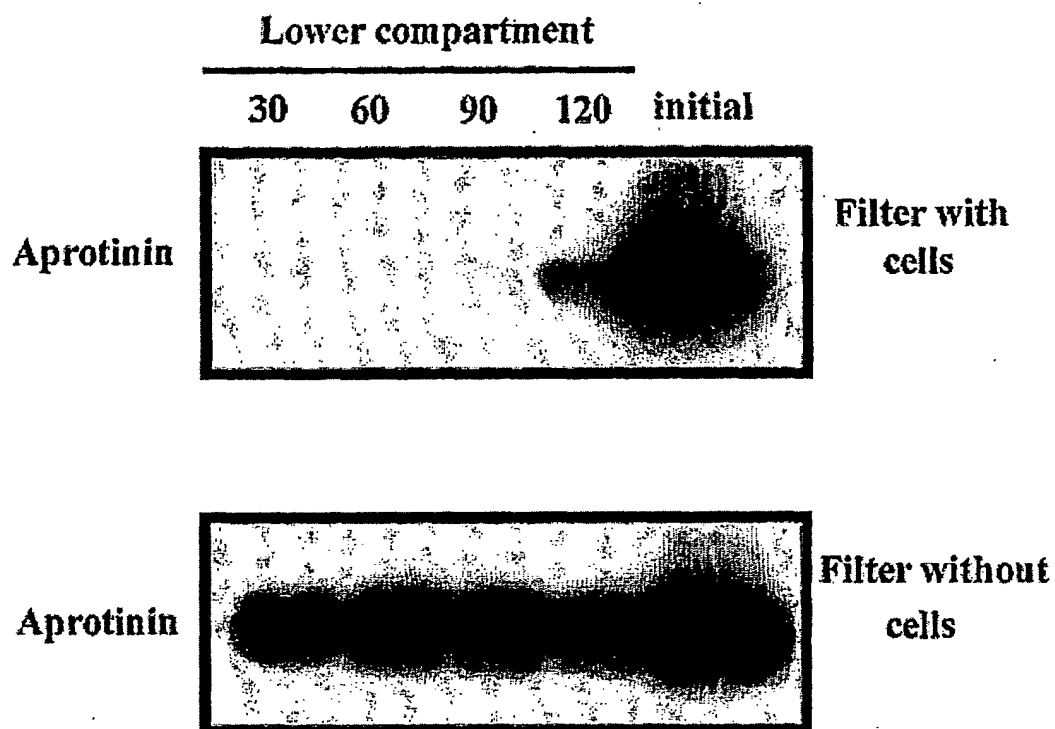


FIG. 4

5/20

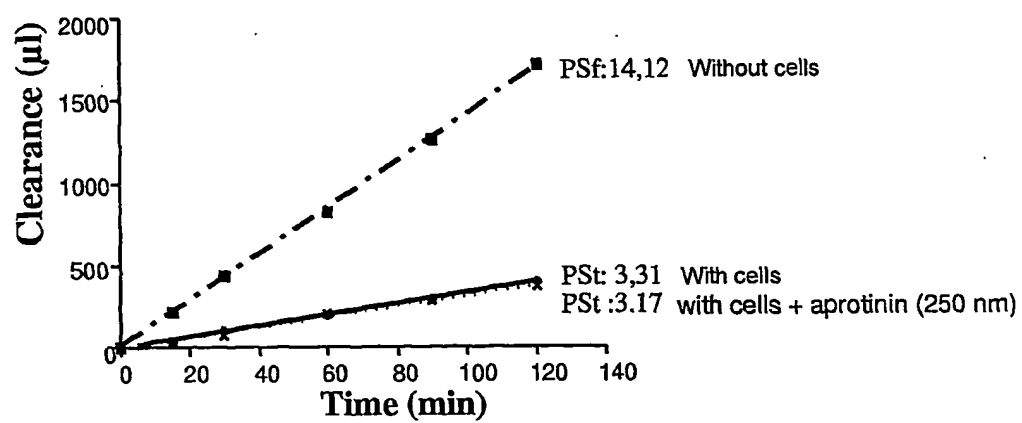


FIG. 5

6/20

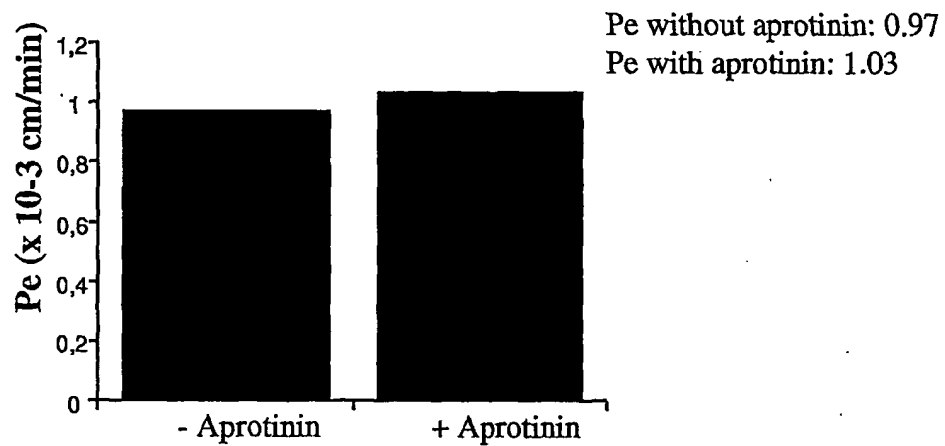


FIG. 6

7/20

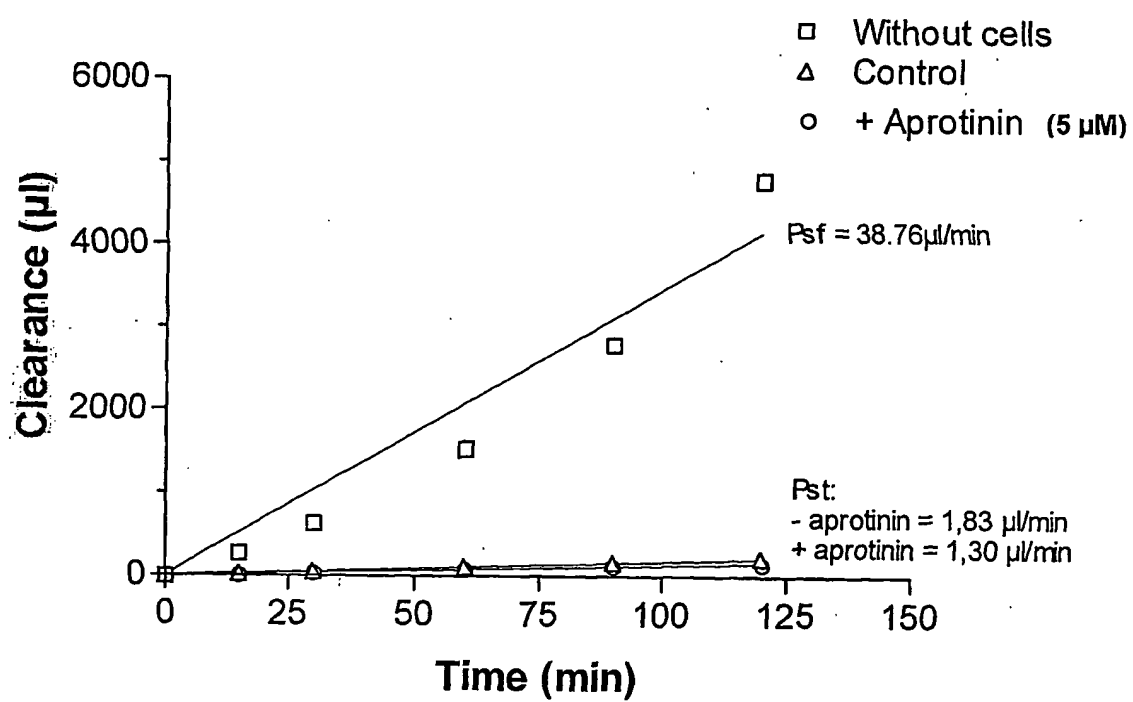


FIG. 7

8/20

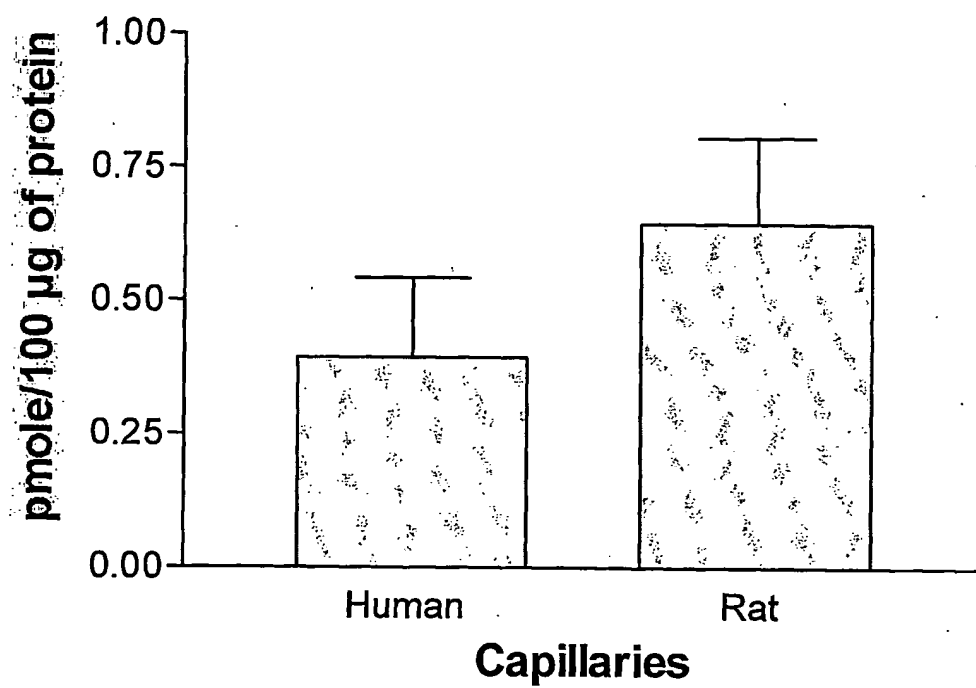


FIG. 8

9/20

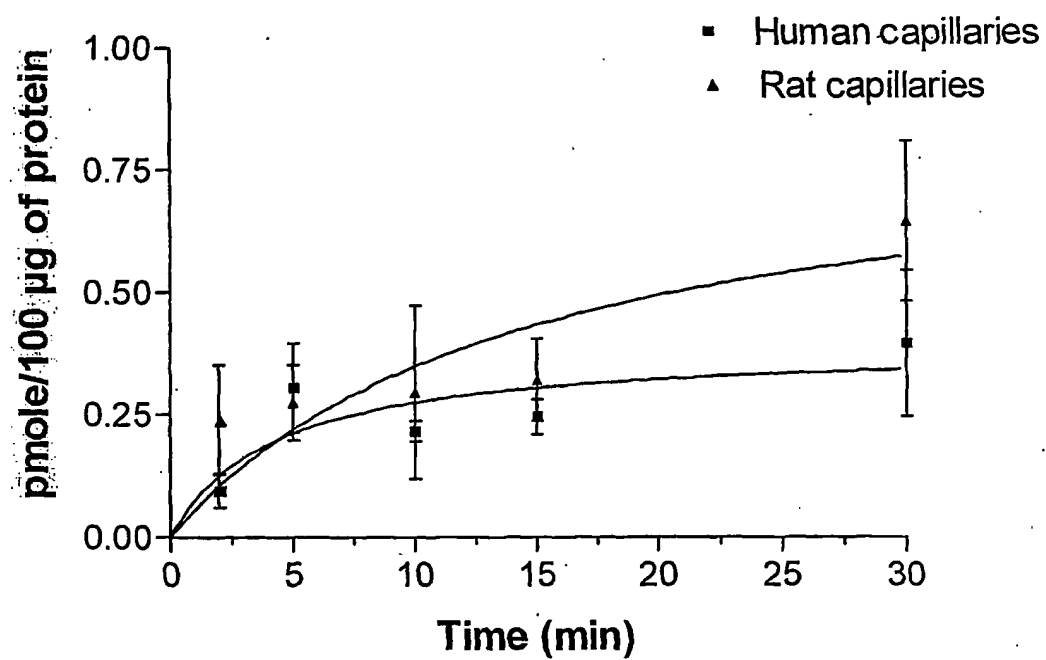


FIG. 9

10/20

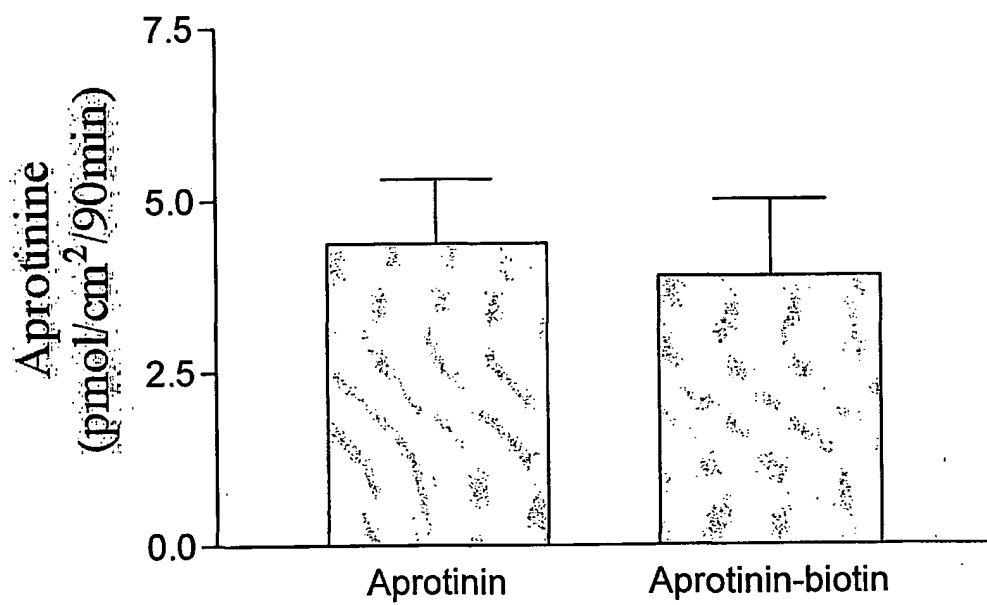


FIG. 10

11/20

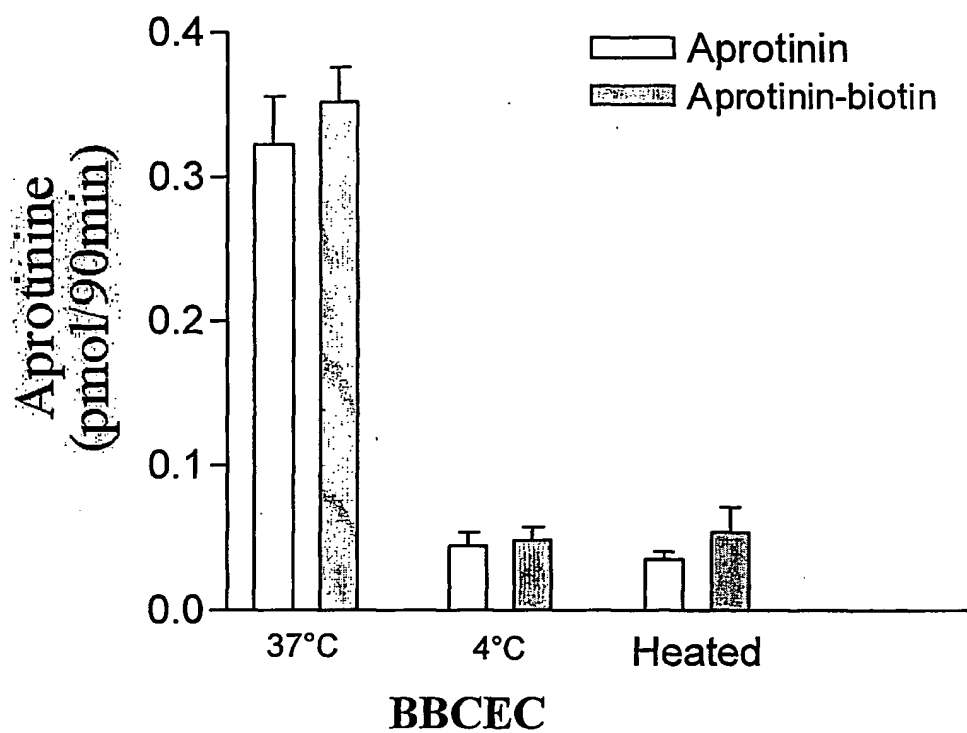


FIG. 11

12/20

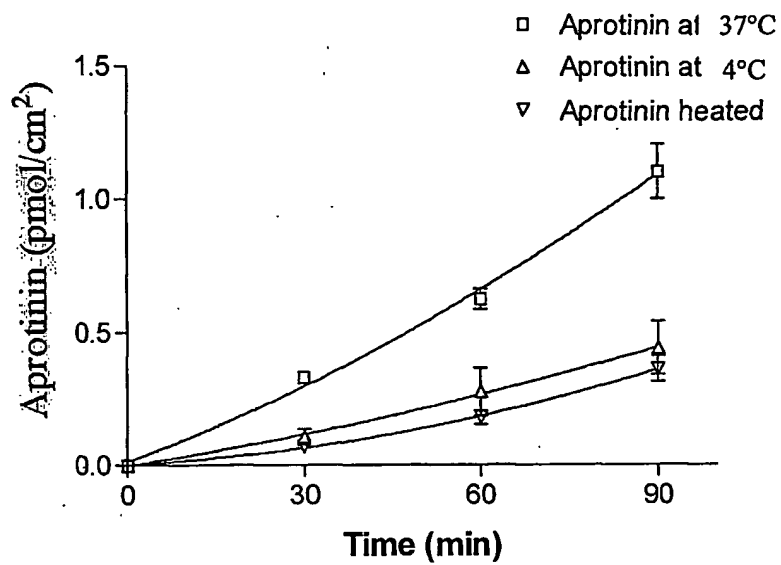


Fig. 12A

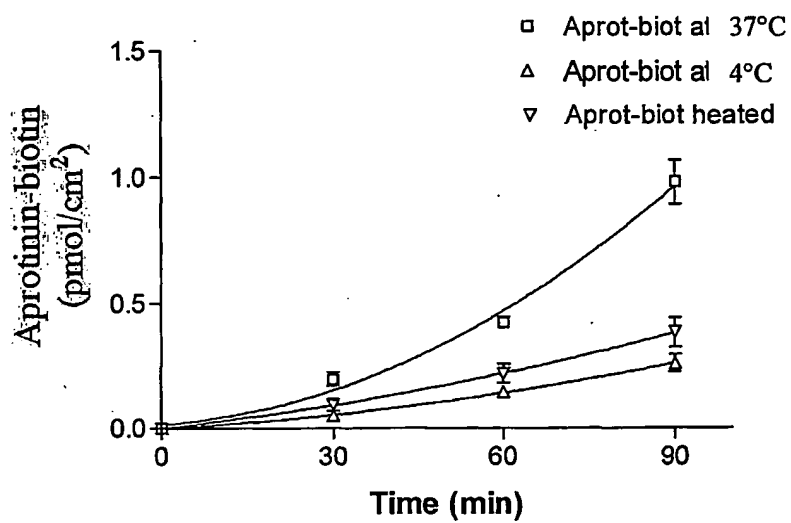


FIG. 12B

13/20

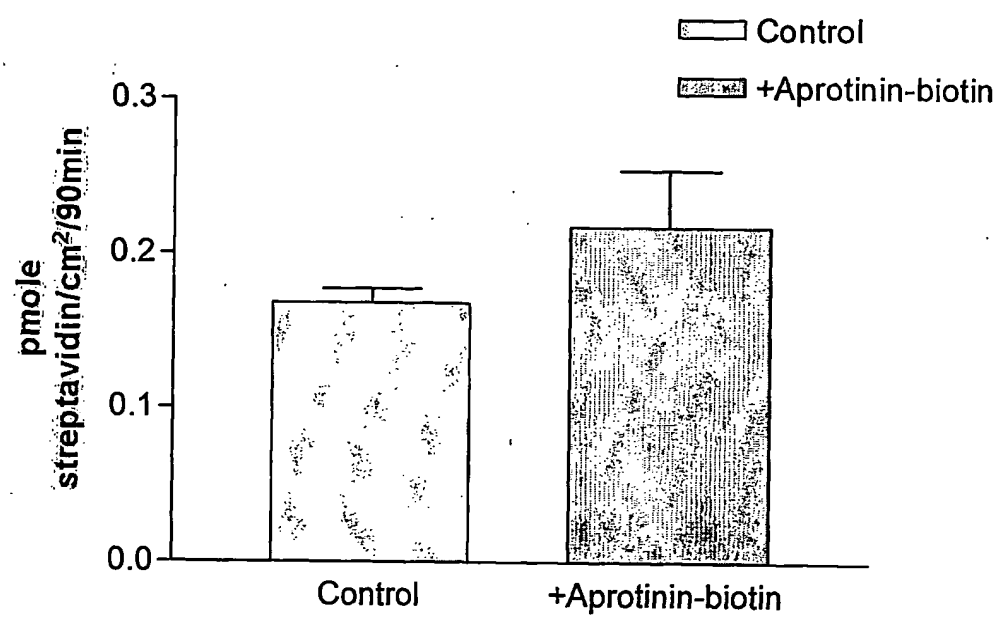


FIG. 13

14/20

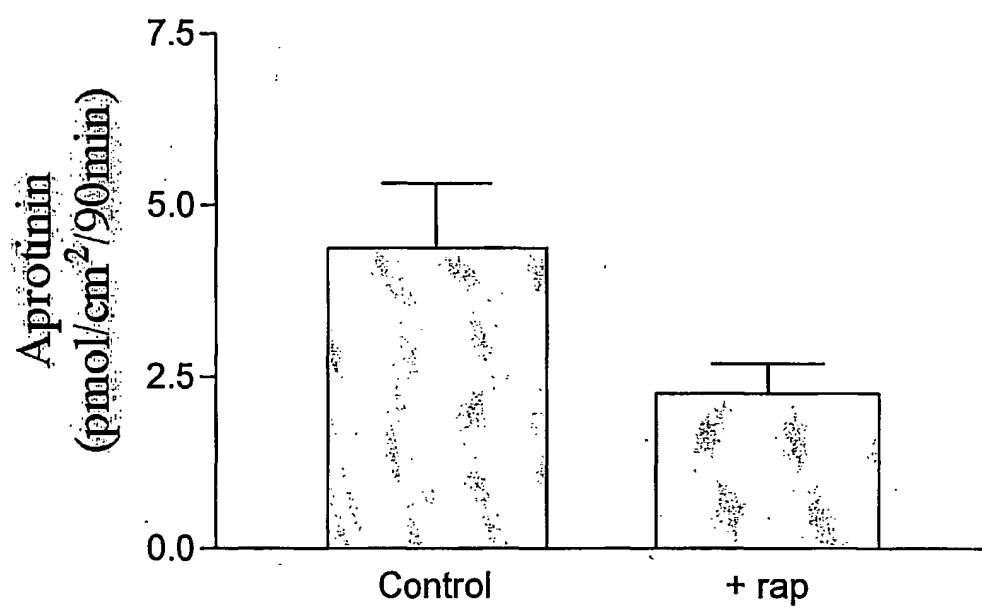
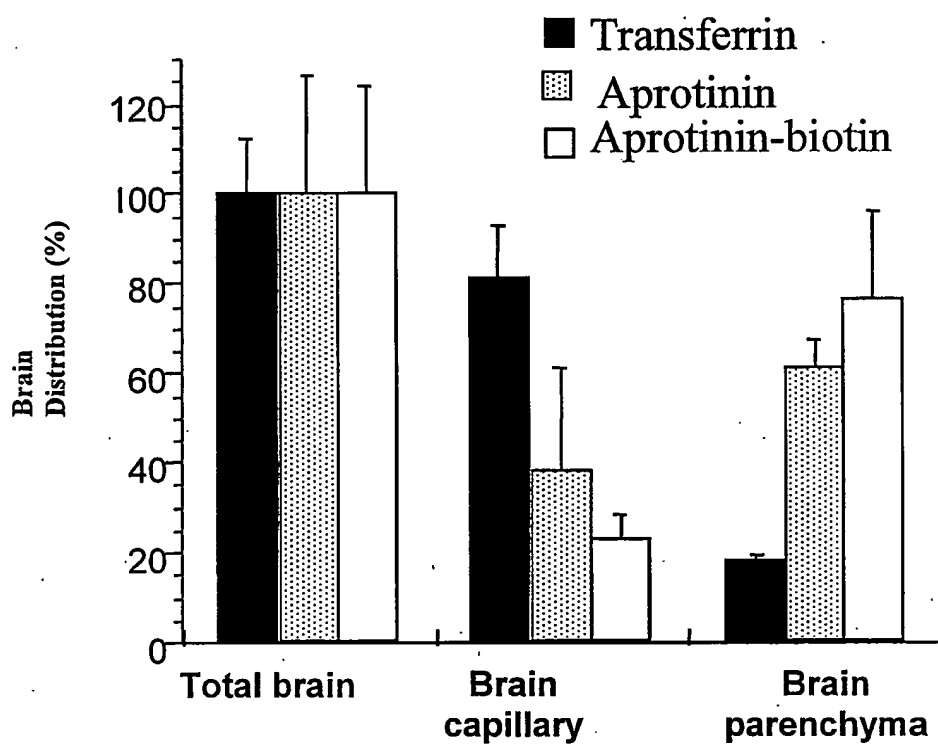


FIG. 14

15/20



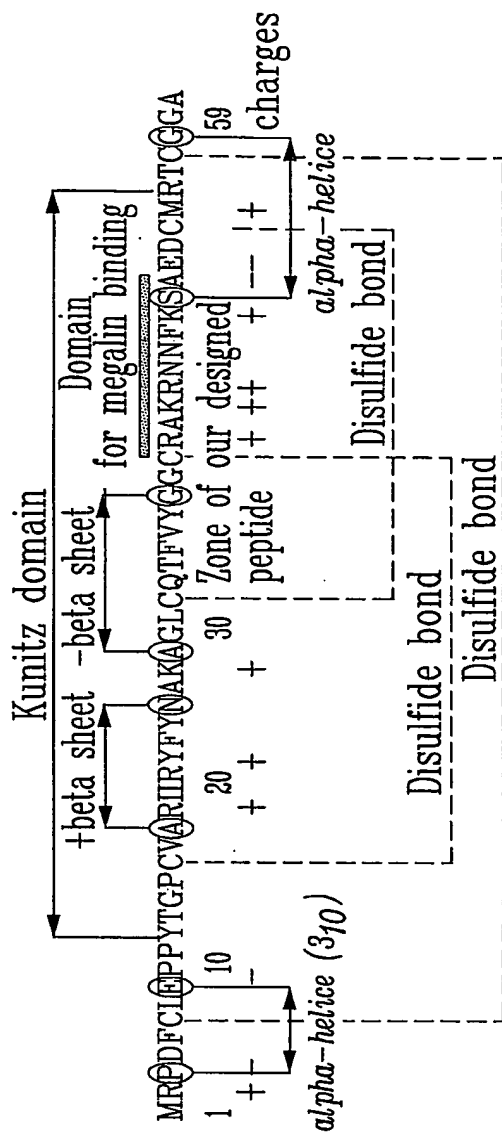
V_D for aprotinin in brain parenchyma = 3 $\mu\text{l}/100\text{ g}$

FIG. 15

16 / 20

Synthetic-Aprotinin Sequence (net charge + 5)
Protein of 59 amino-acids, 6500 Da:

FIG. 16



- * Zone of our designed peptides
- * 4 nucleophiles amines=4 potential sites of conjugaison (residue 1,27,42,47)
- * 6 cysteines engaged in disulfides bonds
- * 2 alpha-helices and 2 beta sheets

18/20

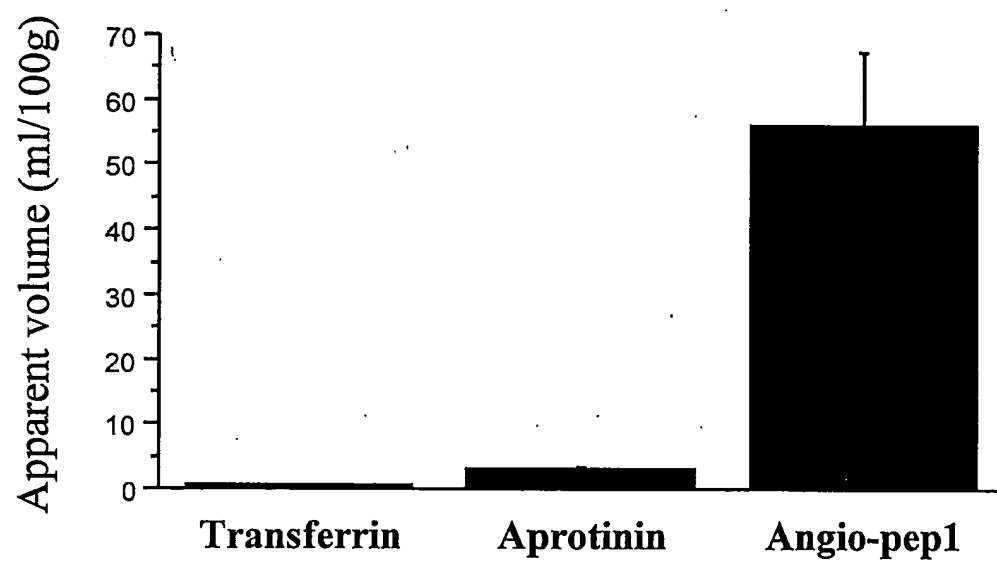


FIG. 18

19/20

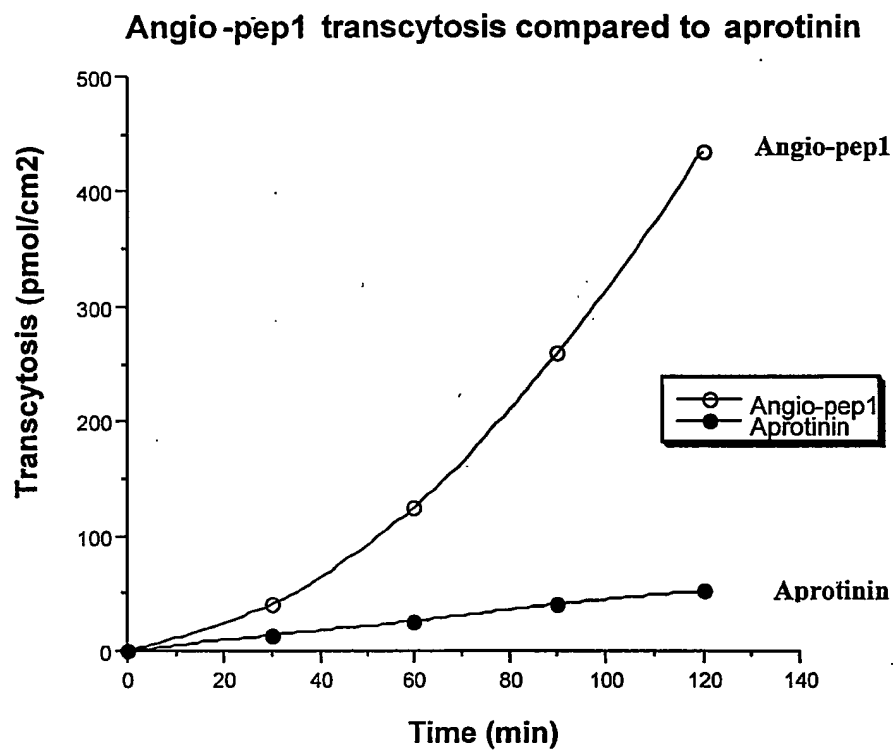


FIG. 19

20/20

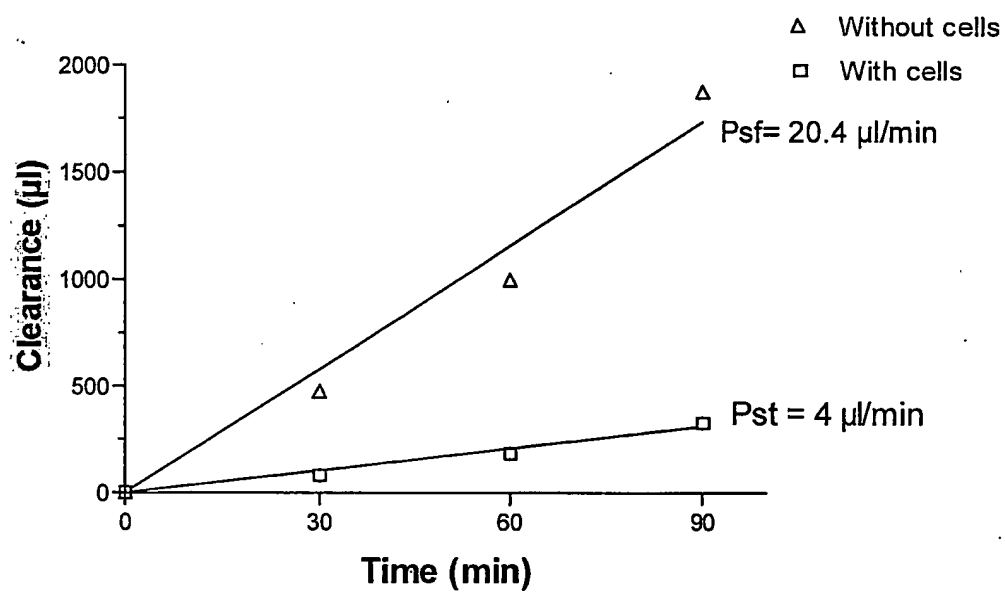


FIG. 20

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
22 July 2004 (22.07.2004)

PCT

(10) International Publication Number
WO 2004/060403 A3

(51) International Patent Classification⁷: A61K 47/48, A61P 25/00

(74) Agent: OGILVY RENAULT; Suite 1600, 1981 McGill College Avenue, H3A 2Y3 Montréal, Québec, CA (CA).

(21) International Application Number:
PCT/CA2004/000011

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(22) International Filing Date: 5 January 2004 (05.01.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/437,986 6 January 2003 (06.01.2003) US

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(71) Applicant (for all designated States except US): TRANSFERT PLUS [CA/CA]; 550 Sherbrooke West, Suite 100, H3A 1B9 Montréal, Québec (CA).

(72) Inventors; and

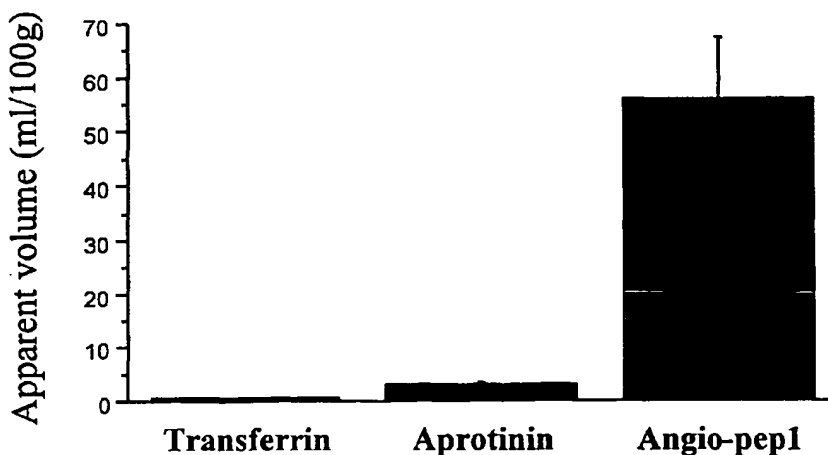
(75) Inventors/Applicants (for US only): BÉLIVEAU, Richard [CA/CA]; 266 Wilson, H3E 1L8 Montréal, Québec, CA (CA). DEMEULE, Michel [CA/CA]; 3557 Archambault, J4M 2W8 Longueuil, Québec, CA (CA).

Published:

— with international search report

[Continued on next page]

(54) Title: APROTININ AND ANGLOS AS CARRIERS ACROSS THE BLOOD-BRAIN BARRIER



(57) Abstract: The present invention relates to improvements in the field of drug delivery. More particularly, the invention relates to a non-invasive and flexible method and carrier for transporting a compound or drug across the blood-brain barrier of an individual. In particular the present invention relates to a carrier for transporting an agent attached thereto across a blood-brain barrier, wherein the carrier is able to cross the blood-brain barrier after attachment to the agent and thereby transport the agent across the blood-brain barrier. The present invention relates to improvements in the field of drug delivery. More particularly, the invention relates to a non-invasive and flexible method and carrier for transporting a compound or drug across the blood-brain barrier of an individual. In particular the present invention relates to a carrier for transporting an agent attached thereto across a blood-brain barrier, wherein the carrier is able to cross the blood-brain barrier after attachment to the agent and thereby transport the agent across the blood-brain barrier.



— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(88) **Date of publication of the international search report:**
18 November 2004

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA2004/000011

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48 A61P25/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|---|
| X | DE 199 53 696 A (CHERKASKY ALEXANDER) 10 May 2001 (2001-05-10) column 1, lines 15-32; claim 3; figure 1 ----- -/-- | 1-4, 7-21, 24-38, 41-53, 56-67, 70-78, 80-102 |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

19 May 2004

Date of mailing of the international search report

30. 09. 2004

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Gonzalez Ramon, N

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA2004/000011

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|---|
| X | SHIMURA T ET AL: "TRANSPORT MECHANISM OF A NEW BEHAVIORALLY HIGHLY POTENT ADRENOCORTICOTROPIC HORMONE (ACTH) ANALOG, EBIRATIDE, THROUGH THE BLOOD-BRAIN BARRIER" JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS, AMERICAN SOCIETY FOR PHARMACOLOGY AND, US, vol. 258, no. 2, 1991, pages 459-465, XP008030272 ISSN: 0022-3565 abstract; figure 1 | 1,2,4, 7-19,21, 24-36, 38, 41-51, 53, 56-65, 67, 70-77, 80-102 |
| Y | ----- DEMEULE M ET AL: "HIGH TRANSCYTOSIS OF MELANOTRANSFERRIN (P97) ACROSS THE BLOOD-BRAIN BARRIER" JOURNAL OF NEUROCHEMISTRY, NEW YORK, NY, US, vol. 83, no. 4, November 2002 (2002-11), pages 924-933, XP001188983 ISSN: 0022-3042 see discussion abstract | 1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102 |
| Y | ----- SEIDEL G ET AL: "EFFECTS OF TRASYLOL ON THE BLOOD-BRAIN BARRIER IN RATS" NAUNYN-SCHMIEDEBERG'S ARCHIVES OF PHARMACOLOGY, SPRINGER, BERLIN, DE, vol. 284, no. 4, 1974, page R73, XP008030270 ISSN: 0028-1298 abstract | 1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102 |
| Y | ----- MARTEL ET AL: "Transport of apolipoproteins E and J at the blood - brain barrier. Relevance to Alzheimer's disease" STP PHARMA SCIENCES, PARIS, FR, vol. 7, no. 1, 1997, pages 28-36, XP002090769 ISSN: 1157-1489 abstract | 1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102 |
| P,X | ----- WO 03/009815 A (KENNARD MALCOLM L ;YANG JOSEPH (CA); DEMEULE MICHEL (CA); BELIVEAU) 6 February 2003 (2003-02-06) page 4; figure 17 page 37, line 8; claims 8,25 ----- -/-- | 1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102 |

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA2004/000011

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|---|
| A | WO 02/33090 A (PROCYON BIOPHARMA INC) 25 April 2002 (2002-04-25) page 1, lines 42,43; claims 12,22,36,46,52,58,75,82,88; example 18 ----- | 1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102 |
| A | GUILLOT F L ET AL: "ANGIOTENSIN PEPTIDE REGULATION OF BOVINE BRAIN MICROVESSEL ENDOTHELIAL CELL MONOLAYER PERMEABILITY" JOURNAL OF CARDIOVASCULAR PHARMACOLOGY, NEW YORK, NY, US, vol. 18, no. 2, 1991, pages 212-218, XP008030278 ISSN: 0160-2446 abstract page 217, column 2 ----- | 1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102 |
| P,Y | KOBAYASHI H ET AL: "THE PROTEASE INHIBITOR BIKUNIN, A NOVEL ANTI-METASTATIC AGENT" BIOLOGICAL CHEMISTRY, XX, XX, vol. 384, no. 5, 1 May 2003 (2003-05-01), pages 749-754, XP008030275 ISSN: 1431-6730 abstract; figure 2 ----- | 1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2004/000011

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4, 7-21, 24-38, 41-53, 56-67, 70-78, 80-102 (all partially), 5, 22, 39
54, 68

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 5, 22, 39, 54, 68 complete; 1-4, 7-21, 24-38, 41-53, 56-67, 70-78, 80-102 partially

Carrier for transporting an agent attached thereto across the blood brain barrier wherein the agent is anticancer agent paclitaxel. Conjugate comprising the carrier and paclitaxel, pharmaceutical composition and use of the same for neurological disease (brain tumour, brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and obesity).

2. claims: 1-4, 6-21, 23-38, 40-53, 55-67, 69-79, 80-102 partially

Carrier for transporting an agent attached thereto across the blood brain barrier wherein the agent is a green fluorescent protein, a histag protein, and beta galactosidase. Conjugate comprising the carrier and the protein agent, pharmaceutical composition and use of the same for neurological disease (brain tumour, brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and obesity)

3. claims: 1-4, 6-21, 23-38, 40-53, 55-67, 69-79, 80-102 partially

Carrier for transporting an agent attached thereto across the blood brain barrier wherein the agent is a green fluorescent protein, a histag protein, and beta galactosidase. Conjugate comprising the carrier and the protein agent, pharmaceutical composition and use of the same for neurological disease (brain tumour, brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and obesity)

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA2004/000011

| Patent document cited in search report | | Publication date | Patent family member(s) | | Publication date |
|---|---|---------------------|----------------------------|---------------|---------------------|
| DE 19953696 | A | 10-05-2001 | DE | 19953696 A1 | 10-05-2001 |
| ----- | | | | | |
| WO 03009815 | A | 06-02-2003 | CA | 2450073 A1 | 06-02-2003 |
| | | | WO | 03009815 A2 | 06-02-2003 |
| | | | US | 2003129186 A1 | 10-07-2003 |
| ----- | | | | | |
| WO 0233090 | A | 25-04-2002 | CA | 2321256 A1 | 16-04-2002 |
| | | | CA | 2355334 A1 | 16-04-2002 |
| | | | AU | 1369102 A | 29-04-2002 |
| | | | WO | 0233090 A2 | 25-04-2002 |
| | | | CA | 2359650 A1 | 16-04-2002 |
| | | | EP | 1326981 A2 | 16-07-2003 |
| | | | JP | 2004510833 T | 08-04-2004 |
| | | | US | 2003170220 A1 | 11-09-2003 |
| ----- | | | | | |

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER: _____**

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

B4

12 **EUROPÄISCHE PATENTANMELDUNG**

21 Anmeldenummer: 90106529.2

51 Int. Cl.⁵: **C07K 7/10, A61K 37/64,**
C12N 15/15

22 Anmeldetag: 05.04.90

30 Priorität: 18.04.89 DE 3912638

43 Veröffentlichungstag der Anmeldung:
24.10.90 Patentblatt 90/43

84 Benannte Vertragsstaaten:
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

71 Anmelder: **BAYER AG**

D-5090 Leverkusen 1 Bayerwerk(DE)

72 Erfinder: **Bruns, Wolfgang, Dr.**
Kaiser-Wilhelm Allee 37
D-5600 Wuppertal 1(DE)
Erfinder: **Schnabel, Eugen, Dr.**
Schimmelweg 6
D-5600 Wuppertal 11(DE)
Erfinder: **von Wilcken-Bergmann, Brigitte, Dr.**
Mauenheimerstrasse 70
D-5000 Koeln 80(DE)

54 **Gentechnologisch hergestellte Homologe des Alzheimer-Protease-Inhibitors, Wirtstämme sowie Expressionsvektoren für ihre Herstellung für ihre Verwendung als Arzneimittel.**

57 Die vorliegende Erfindung betrifft Homologe des Alzheimer Protease Inhibitors (API), deren Aminosäuresequenzen sowie das Verfahren zu ihrer Herstellung mit gentechnologischen Methoden und ihre Verwendung als Arzneimittel.

EP 0 393 431 A1

Gentechnologisch hergestellte Homologe des Alzheimer Protease Inhibitors, Wirtstämme sowie Expressionsvektoren für ihre Herstellung und ihre Verwendung als Arzneimittel

Die vorliegende Erfindung betrifft Homologe des Alzheimer Protease Inhibitors (API), deren Aminosäuresequenzen sowie das Verfahren zu ihrer Herstellung mit gentechnologischen Methoden und ihre Verwendung als Arzneimittel.

Amyloid-Protein-Aggregate wurden in neuritischen Plaques und cerebrovaskulären Ablagerungen bei Patienten mit Alzheimer'scher Krankheit gefunden (C.L. Masters et al., Proc. Nat. Acad. Sci USA 82, 4245-4249, 1985). Molekularbiologische Untersuchungen der entsprechenden cDNA aus humanem Gehirngewebe zeigten, daß das Amyloid-Protein Bestandteil eines Precursorproteins von 695 Aminosäuren ist (J. Kang et al., Nature 325, 733-736; 1987). Weiterführende Untersuchungen an cDNAs aus peripherem Gewebe führten zur Entdeckung von verwandten cDNA-Spezies, die sich durch ein zusätzliches DNA-Segment von 168 Basenpaaren (bp) (P. Ponte et al., Nature 331, 525-527 bzw. R.E. Tanzi et al., Nature 331, 528-530; 1988) bzw. 225 bp (N. Kitaguchi et al., Nature 331, 530-532; 1988) im Gen des Amyloid-Precursorproteins auszeichnen.

Die von dem 168 bzw. 225 bp DNA-Segment abgeleitete Aminosäuresequenz im Leseraster des Amyloid-Precursorproteins zeigt starke Homologie mit Proteaseinhibitoren vom Kunitztyp, wie z.B. den inhibitorisch aktiven Domänen des Inter- α -Trypsin-Inhibitors (ITI) oder dem basischen pankreatischen Trypsin-Inhibitor (BPTI bzw. Aprotinin). Kitaguchi et al konnten Trypsin-inhibitorische Aktivität nach Transfektion von COS-1 Zellen mit der um 225 bp längeren cDNA des Amyloid-Precursors nachweisen. Die Funktion und Bedeutung des beschriebenen Alzheimer Protease Inhibitors (API) für die Entstehung des Amyloid-Plaques und für die Pathogenese der Alzheimer'schen Krankheit ist nicht bekannt.

Proteaseinhibitoren wie das dem API verwandte Aprotinin (Basischer Pankreatischer Trypsin Inhibitor aus Rinderorganen) besitzen eine breite inhibitorische Spezifität und werden seit vielen Jahren als Arzneimittel in der Therapie von Krankheiten verwendet, die sich unter anderem durch einen Mangel an natürlichen Proteaseinhibitoren auszeichnen (vgl. H. Fritz und G. Wunderer, Arzneimittelforschung 33, 479-494; 1983).

Zahlreiche Krankheiten sind nun aber bedingt durch eine Freisetzung von Enzymen, die nicht durch Inhibitoren wie Aprotinin gehemmt werden können. Dazu gehören lysosomale Enzyme wie z.B. die Granulozytenelastase. Normalerweise wird diese bei der Ausschüttung in den extrazellulären Raum inaktiviert durch Komplexbildung mit körpereigenen Inhibitoren wie α_1 -Proteaseinhibitor (α_1 -PI) (J. Travis und G.S. Salvesen, Ann. Rev. Biochem. 655-709; 1983) durch Antileukoprotease (HUS-1) (H. Schissler et al. in: Neutral Proteases of Human Polymorphonuclear Leukocytes (1978) 195-207; K. Havemann und A. Janoff Hrgb., Urban + Schwarzenberg, Baltimore) oder durch α_2 -Makroglobulin (α_2 -M) (G. Salvesen, D. Virca und J. Travis, Ann N.Y. Acad. Sci. 421, 316-326; 1983). Ein hereditärer α_1 -PI-Mangel bzw. die oxidative Inaktivierung des Inhibitors (J. Travis und G.S. Salvesen, Ann. Rev. Biochem. 655-709; 1983) oder eine massive Enzymausschüttung, insbesondere von Granulozytenelastase, führen zu einem extensiven Abbau von Bindegewebe und humoralen Funktionsproteinen mit ernsthaften klinischen Symptomen wie Lungenemphysem, Schocklunge, Adult Respiratory Distress Syndrome, Gerinnungsstörungen sowie Nieren- und Leber-Versagen (vgl. M. Jochum et al. in: neue Wege in der Entzündungsdiagnostik, PMN-Elastase (1985), GIT-Verlag, Darmstadt; W.W. McGuire et al., J. Clin. Invest. 69, 543; 1982 sowie C.T. Lee et al., N. Engl. J. Med. 304, 192-196; 1981). Auch bei akuten und chronischen Entzündungen, z.B. bei der rheumatoiden Arthritis ist die verhängnisvolle Rolle der Granulozytenelastase gesichert (K. Klesiek et al. in: Neue Wege in der Entzündungsdiagnostik, PMN-Elastase (1985) 71-82, GIT-Verlag, Darmstadt).

In experimentellen Sepsis- und Emphysem-Modellen wurde die therapeutische Wirksamkeit von synthetischen Elastaseinhibitoren (J.C. Powers, Ann. Rev. Respir. Dis. 127, 554-558; 1983) sowie dem gentechnologisch hergestellten Elastaseinhibitor Eglin c (H.P. Schnebli et al., Europ. J. Respir. Dis 66 Suppl. 66-70; 1985) nachgewiesen. Vor allem in der Langzeittherapie bietet jedoch der Einsatz eines stabilen und oxidationsunempfindlichen Elastaseinhibitors humaner Provenienz wegen des fehlenden Allergisierungsrisikos und der besseren Verträglichkeit erhebliche Vorteile.

Es wurde gefunden, daß das Hemmspektrum von API durch Aminosäureaustausch in Position 13 (P1) (Abb. 1) mittels rekombinanter DNA-Technologie selektiv verändert werden kann und daß in Kombination mit Aminosäureaustauschen in Position 15 und 37 spezifische Inhibitoren gegen Serinproteasen wie z.B. Elastase, Kallikrein und Cathepsin G erhältlich sind.

Darüber hinaus sollte wegen des geringen Molekulargewichts von API-Homologen im Komplex mit der Leukozytenelastase eine effiziente Ausscheidung von Inhibitor und freigesetzter Elastase über die Nieren erfolgen.

Die Tatsache, daß das API-Protein humanen Ursprungs ist sowie sein niedriges Molekulargewicht lassen in der klinischen Anwendung von API-Homologen geringe Probleme hinsichtlich der Verträglichkeit und des Risikos allergischer Komplikationen erwarten.

- Ein weiterer Vorteil von API-Nomologen nach Aminosäureaustausch von Met in Position 15 ist, im Vergleich zu α_1 -P1 und Antileukoprotease, deren Unempfindlichkeit gegenüber der oxidativen Inaktivierung bei entzündlichen Prozessen, bei denen stark oxidative Substanzen produziert und freigesetzt werden. Dadurch sollten, im Vergleich zu α_1 -P1 und Antileukoprotease, geringere Dosen von API-Homologen notwendig sein, um einen vergleichbaren Schutz gegen Proteasen zu erreichen.

- Ziel der Erfindung ist es, pharmakologisch nützliche Peptide herzustellen, die Proteaseinhibitionsaktivität mit veränderter Selektivität und verbesserter inhibitorischer Effizienz besitzen. Diese gentechnologisch hergestellten Peptide beinhalten die Aminosäuresequenz des Alzheimer Protease Inhibitors (API), wobei mindestens eine Aminosäure der natürlichen Sequenz durch eine andere natürliche Aminosäure ersetzt worden ist. Die erfindungsgemäßen Inhibitoren können außerdem veränderte N-terminale und/oder C-terminale Aminosäuresequenzen besitzen, ohne daß hierdurch ihre inhibitorische Spezifität verändert wäre. Darunter sind zu verstehen: Deletionen einer oder mehrerer Aminosäuren oder Additionen von Aminosäuren, wie z.B. von Signalpeptiden oder von Met am NH_2 -Terminus bzw. Addition von Oligopeptiden als Linkersequenzen am COOH - oder NH_2 -Terminus, die konstruktionsbedingt sind oder mit deren Hilfe eine höhere Expression oder eine Verbesserung der Proteinreinigung erreicht werden kann. Desgleichen sind unter erfindungsgemäßen API-Homologen Inhibitoren mit Aminosäureaustauschen an Positionen von sauren (Asp, Glu) bzw. basischen (Arg, Lys) Aminosäureresten (ausgenommen P_1) zu verstehen, mit dem Ziel, bei unveränderter inhibitorischer Spezifität die Azidität und damit die pharmakokinetischen Eigenschaften des Inhibitors gezielt zu verändern. Gleiches kann auch durch Substitution neutraler Aminosäurereste in flexiblen Regionen des API durch basische oder saure Aminosäuren erzielt werden, beispielsweise Ersatz von Position 37-39 durch Arg-Ala-Lys, wodurch zusätzlich 2 basische Aminosäuren im API eingeführt werden können, ohne die Spezifität zu verändern.

Ganz allgemein werden unter erfindungsgemäßen Inhibitoren auch solche API-Varianten verstanden, die durch einen Aminosäureaustausch in Position 13 zu Inhibitoren der Leukozytenelastase, des Kallikreins oder des Cathepsin G wurden und zusätzlich einen, zwei oder auch mehrere weitere Aminosäureaustausche tragen, die diese Hemmspezifität nicht verändern.

- In den erfindungsgemäßen, gentechnologisch hergestellten Homologen des API können neben einem veränderten Aminosäurerest im aktiven Zentrum P_1 (Position 13) zusätzlich ein oder mehrere Aminosäurereste in anderen Positionen im Molekül ausgetauscht sein. Solche bevorzugten Positionen für Austausche sind

Position 13 (P_1 = aktives Zentrum):

- Val, Ile, Leu, Ala, Met, Asn, Gln, Phe, Tyr oder Trp

Position 15:

Leu, Gly, Ala, Phe, Arg, Val, Ile, Ser, Thr, Asp, Glu, Asn, Gln oder Tyr

Position 37:

Arg, Val, Ile, Leu, Met, Asn, Gln, Asp, Glu, Phe, Ser oder Thr

- Position 50:

Val, Ile, Leu, Gln, Glu, Thr, Asp, Phe, Lys oder Arg.

- Gegenstand der Erfindung sind außerdem die synthetischen Desoxyribonukleinsäuren (DNAs), die für die erfindungsgemäßen API-Homologen kodieren. Der Austausch einzelner Aminosäurereste ist bekanntermaßen durch ortsspezifische Mutagenese oder Synthese des entsprechenden Gens möglich. Methoden zur Expression der heterologen DNA in Mikroorganismen oder in eukaryotischen Zellen sind ebenso bekannt. So wurde gezeigt, daß Aprotinin oder Homologe in rekombinanten Mikroorganismen unter Verwendung synthetischer Gene entweder als Met-Aprotinin (B. v. Wilcken-Bergmann et al, EMBO J., 5, 3219; 1986) oder als Fusionsprotein mit β -Galaktosidase (E.A. Auerswald et al., Europ. Patentanmeldung 238 993 vom 26.3.1986) exprimiert werden können. Es ist weiterhin bekannt, daß Proteaseinhibitoren als Precursorproteine hergestellt werden können, wie z.B. Aprotinin mit der Signalsequenz der alkalischen Phosphatase (S. Anderson et al., Proc. Nat. Acad. Sci. USA 80, 6368; 1983) und der pankreatische, sekretorische Trypsin-Inhibitor (PSTI) mit der OMPA-Signalsequenz (F. Maywald et al., Gene 68, 357-369; 1988).

- Eine andere Möglichkeit der Genexpression in Form von "inclusion bodies" ist für die Herstellung von humanem Aprotinin als Fusionsprotein mit einer Teilsequenz der MS2-DNA-Polymerase gezeigt worden (E. Schnabel et al., EP-A 297 362 bzw. DE-OS 3 724 570). Der gewünschte Proteaseinhibitor ist durch selektive, enzymatische oder chemische Spaltung des Fusionsproteins zugänglich, beispielsweise durch BrCN-Spaltung, wenn die Verknüpfung der Fusionspartner über ein Methionin erfolgt, und wenn gleichzeitig diese Aminosäure in dem Proteaseinhibitor abwesend ist bzw. ausgetauscht werden kann.

Es ist darüber hinaus auch möglich, die erfindungsgemäßen Proteaseinhibitoren in eukaryontischen Organismen zu exprimieren (z.B. Hefen, filamentöse Pilze etc.).

Die vorliegende Erfindung betrifft insbesondere die synthetische DNA-Sequenz in Abb. 2 und deren durch verschiedene Codons derselben Aminosäure abgeleiteten Äquivalente, bei deren Translation die erfindungsgemäßen Inhibitoren erhalten werden.

Abhängig von der Art des Expressionssystems können die erfindungsgemäßen Gene der API-Homologen zusätzlich ein Startcodon für Met oder eine DNA-Sequenz enthalten, die für ein Signalpeptid, ein Linkerpeptid oder ein Fusionsprotein kodiert.

Weiterhin betrifft die Erfindung einen Hefe-Expressionsvektor, in welchen die erfindungsgemäßen DNA-Sequenzen für die beanspruchten API-Homologen inseriert werden und der für die Transformation geeigneter Wirkstämme verwendet wird.

Je nach der Natur des Expressionssystems können die erfindungsgemäßen Gene für die Expression der beanspruchten Proteinaseinhibitoren zusätzlich ein Start-Codon für Met oder eine Nukleotidsequenz vorgeschaltet haben, die für ein Signalpeptid kodiert.

Bevorzugte Fusionspartner für die beanspruchten Proteaseinhibitoren können sekretorische Signalsequenzen von mikrobiellen Proteinen sein, wie z.B. omp A (Ghrayed u.a. EMBO Journal 3, 2437-2442; 1984) oder pho A (Gray u.a., Gene 39, 247-254; 1985). Im Falle der Expression in Hefen kann es die Signalsequenz des mating factors alpha (MF α) sein (Kurjan, Cell 30, 933; 1982).

Die Fusionsproteine werden während der Fermentation in das Periplasma oder Kulturmedium sezerniert und nach an sich bekannten Verfahren aus den Kulturfiltraten isoliert. Bei der Wahl von geeigneten sekretorischen Systemen werden die Proteaseinhibitoren als native Proteine sezerniert. Eine Renaturierung nach der Bromcyan-Spaltung (E. Gross und B. Witkop, J. Amer. Chem. Soc. 83, 1510-1511; 1961) entfällt für den Fall einer korrekten Prozessierung beim Membrandurchtritt. Die erfindungsgemäßen Proteinaseinhibitoren werden aus den Reaktionsgemischen nach an sich bekannten Verfahren gereinigt und charakterisiert.

Eine alternative Möglichkeit der Gen-Expression ist im Falle der erfindungsgemäßen Proteinaseinhibitoren realisierbar, wenn die Fusionsproteine in der Zelle unlöslich in Form von "inclusion bodies" ausgeschieden werden. Solche Einschlüsse von unlöslichen Fusionsproteinen können erzeugt werden z.B. durch Fusionierung der API-Gene mit einer DNA-Teilsequenz der DNA-Polymerase des Bacteriophagen MS2 - E. Remault, P. Stanssens und W. Fiers, Gene 15, 81 (1981) und durch Fusion der Inhibitorgene mit einer Teilsequenz vom cII-Gen des Bacteriophagen Lambda (K. Nagai und H.G. Thorngersen, Nature 309, 810; 1984).

Die Produktion der erfindungsgemäßen Proteaseinhibitoren über Einschlußkörper umfaßt die folgenden Schritte:

1. Anzucht und Kultivierung des Wirtsstammes unter geeigneten Bedingungen;
2. Isolierung der Einschlußkörper aus den Wirtszellen;
3. Aufreinigung der Fusionsproteine;
4. Spaltung der Fusionsproteine;
5. Renaturierung der Inhibitoren;
6. Reinigung und Charakterisierung der Inhibitoren.

Für die Reinigung der löslichen Fusionsproteine kann es vorteilhaft sein, in das abzuspaltende Fusionspeptid gehäuft saure oder basische Aminosäuren einzubauen, so daß die Abtrennung durch Ionenaustauschchromatographie erleichtert wird.

Die Endreinigung der erfindungsgemäßen Proteaseinhibitoren erfolgt nach an sich bekannten Verfahren, wie z.B. Gelfiltration, Ionenaustauschchromatographie oder Affinitätschromatographie und Elektrophorese.

Derzeitig kennt man eine große Zahl von verschiedenen Mikroorganismen, die für die Transformation geeignet sind und die in Kulturen oder Fermentationsbrühen gezüchtet werden können. Bevorzugte Organismen zur Transformation schließen Bakterien, Hefen und Pilze ein.

Die vorliegende Erfindung umfaßt pharmazeutische Zubereitungen, die außer nicht-toxischen, inerten, pharmazeutisch geeigneten Exzipienten eine oder mehrere Verbindungen gemäß der Erfindung umfassen oder aus einer oder mehreren aktiven Verbindungen gemäß der Erfindung bestehen, sowie Verfahren zur Herstellung dieser Zubereitungen.

Die vorliegende Erfindung umfaßt auch pharmazeutische Zubereitungen in Dosiseinheiten. Dies bedeutet, daß die Zubereitungen in Form individueller Teile vorliegen, z.B. als Tabletten, beschichtete Tabletten, Kapseln, Pillen, Suppositorien und Ampullen, deren Gehalt an Wirkstoff einer Fraktion oder einem Vielfachen einer individuellen Dosis entspricht. Die Dosiseinheiten können z.B. enthalten: ein, zwei, drei oder vier individuelle Dosen oder eine Hälfte, ein Drittel oder ein Viertel einer individuellen Dosis. Eine individuelle Dosis enthält vorzugsweise die Menge an Wirkstoff, die bei einer Verabreichung gegeben wird, und die

gewöhnlich dem Ganzen, der Hälfte oder einem Drittel oder einem Viertel der Tagesdosis entspricht.

Unter nicht-toxischen, inerten, pharmazeutisch geeigneten Exzipienten sind zu verstehen: feste, halbflüssige oder flüssige Verdünner, Füller und Formulierungshilfsmittel aller Art.

Tabletten, beschichtete Tabletten, Kapseln, Pillen, Granalien, Suppositorien, Lösungen, Suspensionen und Emulsionen, Pasten, Salben, Gele, Cremes, Lotionen, Pulver und Sprays können als bevorzugte pharmazeutische Zubereitungen genannt werden.

Tabletten, beschichtete Tabletten, Kapseln, Pillen und Granalien können den Wirkstoff oder die Wirkstoffe zusammen mit üblichen Exzipienten, wie (a) Füller und Streckmittel, z.B. Stärken, Lactose, Sucrose, Glucose, Mannit und Siliziumoxid, (b) Bindemittel, z.B. Carboxymethylcellulose, Alginate, Gelatine und Polyvinylpyrrolidon, (c) anfeuchtende Mittel, z.B. Glycerin, (d) disintegrierende Mittel, z.B. Agar-Agar, Kalziumcarbonat und Natriumcarbonat, (e) Lösungsverzögerer, z.B. Paraffin und (f) Absorptionsbeschleuniger, z.B. quaternäre Ammoniumverbindungen, (g) Benetzungsmittel, z.B. Cetylalkohol und Glycerinmonostearat, (h) Absorbentien, z.B. Kaolin und Bentonit und (i) Schmiermittel, z.B. Talk, Kalzium- und Magnesiumstearat und feste Polyethylenglykole, oder Gemische der unter (a) bis (i) aufgeführten Substanzen, enthalten.

Die Tabletten, beschichteten Tabletten, Kapseln, Pillen und Granalien können mit üblichen Überzügen und Umhüllungen versehen werden, die gegebenenfalls Opazifizierungsmittel enthalten; sie können eine Zusammensetzung aufweisen, so daß die Freisetzung des Wirkstoffes oder der Wirkstoffe nur, oder vorzugsweise, in einem bestimmten Teil des Intestinaltraktes, gegebenenfalls in verzögerter Form, erfolgt, wobei Beispiele für geeignete Zubereitungen zum Einbetten polymere Substanzen und Wachse bilden.

Die aktive Verbindung oder Verbindungen, gegebenenfalls zusammen mit einem oder mehreren der vorstehend genannten Exzipienten, kann bzw. können auch in mikroverkapselter Form vorliegen.

Suppositorien können außer dem Wirkstoff bzw. den Wirkstoffen enthalten: übliche wasserlösliche oder wasserunlösliche Exzipienten, z.B. Polyethylenglykole, Fette, z.B. Kakaofett und höhere Ester (z.B. C₁₄-Alkohol mit C₁₆-Fettsäure) oder Gemische dieser Substanzen.

Salben, Pasten, Cremes und Gele können zusätzlich zu dem Wirkstoff oder den Wirkstoffen übliche Exzipienten enthalten, z.B. tierische und pflanzliche Fette, Wachse, Paraffine, Stärke, Tragacanth, Cellulose-derivate, Polyethylenglykole, Silikone, Bentonite, Siliziumdioxid, Talk und Zinkoxid, oder Gemische dieser Substanzen.

Pulver und Sprays können außer dem Wirkstoff oder den Wirkstoffen übliche Exzipienten enthalten, z.B.: Lactose, Talk, Siliziumdioxid, Aluminiumhydroxid, Kalziumsilikat und Polyamidpulver, oder Gemische dieser Substanzen. Sprays können außerdem die üblichen Treibmittel enthalten, z.B. Chlorfluorkohlenwasserstoffe.

Lösungen und Emulsionen können außer dem Wirkstoff oder den Wirkstoffen übliche Exzipienten umfassen, wie Lösungsmittel, solubilisierende Agentien und Emulgiermittel, z.B. Wasser, Ethylalkohol, Isopropylalkohol, Ethylcarbonat, Ethylacetat, Benzylalkohol, Benzylbenzoat, Propylenglykol, 1,3-Butylenglykol, Dimethylformamid, Öle, insbesondere Baumwollsaamenöl, Erdnußöl, Maiskeimöl, Olivenöl, Rizinusöl und Sesamöl, Glycerin, Glycerinformal, Tetrahydrofurfurylalkohol, Polyethylenglykole und Fettsäureester von Sorbitan, oder Gemische dieser Substanzen.

Zur parenteralen Verabreichung können die Lösungen und Emulsionen auch in einer sterilen Form vorliegen, die mit Blut isoton ist.

Suspensionen können zusätzlich zum Wirkstoff oder den Wirkstoffen übliche Exzipienten enthalten, wie flüssige Verdünner, z.B. Wasser, Ethylalkohol oder Propylenglykol, Suspendierungsmittel, z.B. ethoxylierte Isostearylalkohole, Polyoxyethylensorbit und Sorbitanester, mikrokristalline Cellulose, Aluminium-metahydroxid, Bentonit, Agar-Agar und Tragacanth, oder Gemische dieser Substanzen.

Die genannten Formulierungsformen können auch enthalten: Farbstoffe, Konservierungsmittel und Additive, die den Geruch oder Geschmack verbessern, z.B. Pfefferminzöl und Eukalyptusöl, und Süßungsmittel, z.B. Saccharin.

Die therapeutisch wirksamen Verbindungen sollten in den vorstehend genannten pharmazeutischen Zubereitungen vorzugsweise in einer Konzentration von 0,1 bis 99,5 Gew.-%, vorzugsweise von ca. 0,5 bis 95 Gew.-%, bezogen auf das Gesamtgemisch, vorliegen.

Die vorstehend genannten pharmazeutischen Zubereitungen können zusätzlich zu den Verbindungen gemäß der Erfindung auch andere pharmazeutische Wirkstoffe enthalten.

Die vorstehend genannten pharmazeutischen Zubereitungen werden in üblicher Weise nach bekannten Verfahren hergestellt, z.B. durch Vermischen des Wirkstoffes oder der Wirkstoffe mit dem Exzipienten oder den Exzipienten.

Die Wirkstoffe oder die pharmazeutischen Zubereitungen können lokal, oral, parenteral, intraperitoneal und/oder rektal verabreicht werden, vorzugsweise erfolgt dies oral oder parenteral, wie z.B. intravenös oder

intramuskulär.

Im allgemeinen hat es sich sowohl in der Humanmedizin als auch in der Tiermedizin als vorteilhaft erwiesen, den Wirkstoff oder die Wirkstoffe gemäß der Erfindung in den Gesamtmengen von ca. 0,5 bis ca. 500, vorzugsweise von 4 bis 100 mg/kg Körpergewicht alle 24 Stunden, gegebenenfalls in Form von mehreren individuellen Verabreichungen, zu geben, um die besten Ergebnisse zu erzielen. Eine individuelle Verabreichung enthält den Wirkstoff oder die Wirkstoffe gemäß der Erfindung vorzugsweise in Mengen von ca. 1 bis ca. 250, insbesondere 3 bis 60 mg/kg Körpergewicht. Es kann jedoch erforderlich sein, von der genannten Dosis abzuweichen, insbesondere in Abhängigkeit von der Natur und dem Körpergewicht des zu behandelnden Individuums, der Natur und Schwere der Krankheit, der Art der Zubereitung und der Verabreichung der Medizin, und der Zeit oder dem Intervall, über den die Verabreichung stattfindet.

So kann es in einigen Fällen genügen, mit weniger als der vorstehend genannten Menge an Wirkstoff auszukommen, während in anderen Fällen die vorstehend genannte Menge an Wirkstoff überschritten werden muß. Die speziell erforderliche optimale Dosis und die Verabreichungsart der Wirkstoffe kann in einfacher Weise vom Fachmann auf der Basis seines Fachwissens entschieden werden.

Die erfindungsgemäßen Wirkstoffe sind sehr gut zur Behandlung von akuten Entzündungen, wie rheumatoider Arthritis, hereditäres angioneurotisches Ödem, Pneumonie, Pankreatitis und von Schockzuständen einsetzbar. Weiterhin haben die Lys-13- sowie die Arg-13-Varianten eine protektive Funktion bei Dialysen mit Hilfe von künstlichen Membranen und allgemein bei der extrakorporalen Zirkulation (open heart surgery). Klinische Befunde zeigen, daß durch Inhibitoren von Plasma-Kallikrein und Plasmin die durch Fibrinolyse und Koagulation bedingten Blutverluste bei "open heart surgery" vermindert werden können, S. 1289-1291; 1987 und C.F Scott et al, Blood, Vol. 69, 1431-1436; 1987).

Material und Methoden

A. Konstruktion der synthetischen Gene und der rekombinanten Klonierungs- und Expressionsvektoren

Materialien:

Reagentien und Enzyme

Reagentien für molekularbiologische und mikrobiologische Experimente wurden von Biolabs, BRL, Pharmacia, Boehringer (Mannheim), Difco, Merck, Serva Sigma und Biorad bezogen.

Radioisotope

Adenosin-5'- α -[³⁵S]-thiotriphosphat wurde von Amersham Buchler bezogen.

DNA

Plasmid- und Phagen-DNA sowie DNA-Linker für die Gen- und Vektorenkonstruktionen wurden bis auf nachfolgende Ausnahmen von Boehringer, Biolabs, BRL und Pharmacia bezogen.

Ausnahmen:

Plasmid pUR 278: U. Rüther und B. Müller-Hill, EMBO Journal 2, 1791-1794 (1983); Plasmid pEx 31.b.: E. Beck, EMBL Heidelberg.

E.coli-Stämme

E.coli RR1ΔM15 (ATCC Nr. 35102): U. Rüther, Nucleic Acids Res. 10, 5765-5772 (1982).

E.coli 537-W6, Lambda rex: W. Fiers, Universität Gent, Belgien (E. Remault u.a. (1981), Gene 15, 81-93).

Medien und Antibiotika

5

Die Rezepturen für LB-, M9s- und Kappa 1776-Medium entsprechen den Angaben in: T. Maniatis u.a. - Molecular Cloning, Gold Spring Harbor, USA (1982) -. Für Agar-Platten wurde den Medien 15 g Bacto-Agar hinzugefügt. Für die Herstellung von Selektionsmedien wurden folgende Antibiotika verwendet: Chloramphenicol und Kanamycin von Boehringer; Ampicillin und Tetracyclin von Serva.

10

Methoden

Die Routine-Techniken für molekulare Klonierungsexperimente, wie Isolierung und Aufreinigung von Plasmid-DNA, Restriktionsanalysen, Auftrennung und Isolierung von DNA-Fragmenten auf Agarose- und Acrylamidgelen, Transformation von Bakterien etc. sind beschrieben in: T Maniatis u.a., Molecular Cloning, Gold Spring Harbor (1982 bzw. 1988).

Chemische Synthese von DNA-Oligonukleotiden

Die für Konstruktion der Proteaseinhibitorgene benötigten DNA-Oligonukleotide wurden mit einer automatischen DNA-Synthese-Maschine von Applied Biosystems, Model 380, synthetisiert. Die Aufreinigung der einzelsträngigen DNA-Oligonukleotide erfolgte über HPLC-Säulen oder durch präparative Acrylamidgелеlektrophorese.

DNA-Sequenzierung

DNA-Fragmente wurden in pUC-Vektoren kloniert und die DNA-Sequenz nach einem Protokoll von Boehringer Mannheim (Leitfaden zum schnellen und einfachen Plasmid-Sequenzieren; 1986) analysiert.

Standard Expression der API-Homologen

35

Zur Expression der API-Homologen wurden sowohl Sekretionsvektoren bzw. Expressionsvektoren zur Produktion von cytoplasmatischen Einschlußkörpern verwendet, wie beschrieben von F. Maywald et al. Gene 68, 357-369 (1988) bzw. E. Schnabel et al., EP-A 297 362 und DE-OS 3 724 570.

Die rekombinanten Sekretionsvektoren wurden in den E.coli Stamm RR1ΔM15 eingeschleust, der MS2-Polymerase-Vektor in den E.coli Stamm 537.

a) Induktion der Sekretionsvektoren

45 Frische Übernachtskulturen der transformierten E.coli Stämme wurden 1:100 im Medium (3 % beef extract, 1,5 % yeast extract, 0,5 % K_2HPO_4) verdünnt.

Die Kulturen wurden bei 28 °C oder 37 °C und 200 UpM bis zu einer $OD_{550nm} = 1,0$ in Erlenmeyerkolben geschüttelt. Die Induktion des lac-Promoters erfolgte durch Zugabe von 1 mM IPTG (Isopropylthiogalactosid).

50 Nach 16 bis 24 Stunden Induktion bei 37 °C wurden die Kulturen mittels Zentrifugation geerntet (10 Minuten; 15 bis 20.000 g; 4 °C). Bei Verwendung der Sekretionsvektoren wurden die Kulturüberstände anhand der enzyminhibitorischen Aktivität auf den Gehalt der sezernierten API-Homologen getestet (s. Methodenteil B).

55

b) Induktion des λ P_L-MS2-Polymerase Vektors

Die Expression der API-Homologen als Fusionsproteine mit einer Teilsequenz der DNA-Polymerase des

Phagen MS2 erfolgte mit einem pEx-Vektor (E. Beck, EMBL-Heidelberg) unter Kontrolle des P_L-Promoters des Phagen Lambda.

Die Anzucht der transformierten E.coli Stämme (Derivate von Ec 537) erfolgte in 10 ml bzw. 1 L LB-Medium mit 100 µg/ml Ampicillin bei 200 UpM und 28° C. Bei einer Wuchsdichte von OD_{550nm} = 1,0 wurde die Temperatur auf 42° C erhöht (Hitzeinduktion) und die Kulturen für weitere 3 Stunden bei 200 UpM inkubiert.

Die Zellen wurden durch Zentrifugation geerntet (s.o.), in SDS-Probenpuffer aufgeschlossen und Aliquots auf SDS-Polyacrylamidgelen durch Elektrophorese aufgetrennt.

Die in Form von Einschlußkörpern akkumulierten Fusionsproteine wurden durch Färbung der Gele mit Coomassie Blau nachgewiesen und der prozentuale Anteil der Fusionsproteine densitometrisch gemessen.

B) Isolierung und Charakterisierung der erfindungsgemäßen Proteaseinhibitoren

15

Materialien

Enzyme

20

Humane Granulozytenelastase (SE 563) und humanes Cathepsin G (SG 45) wurden von Elastin Products Company Inc., P.O. Box 147, Pacific, Ma. 63069, USA bezogen; Trypsin (Rind) von E. Merck, Darmstadt.

Humanes Plasmakallikrein (80-13-1101) stammt von der Fa. Protogen AG, Weidenmattweg 4, Postfach, CH-4448 Läufelfingen, Schweiz. Humanes Harnkallikrein wurde uns von Dr. R. Geiger (Abtig. f. Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik, Universität München, Nußbaumstr 20, 8000 München 2) überlassen; human Plasmin wurde von KABI, AB, Stockholm gekauft; humanes anionisches und kationisches Trypsin von Dr. K. Ohlsson (Dept. Clin. Chemistry and Surgery, Malmö General Hospital, S-21401 Malmö, Schweden).

30

Substrate

Suc-Ala-Ala-Val-PNA und Meosuc-Ala-Ala-Pro-Val-pNA (HGE) sowie Meosuc-Ala-Ala-Pro-Met-pNA (HCG) wurden von der Fa. Bachem, Feinchemikalien AG, Hauptstr. 144, CH-4416 Bubendorf, Schweiz bezogen; D-Pro-Phe-Arg-pNAx2HCl = S-2302 (Plasmakallikrein), D-Val-Leu-Arg-pNAx2HCl = S-2260 (Organkallikrein) und Pyr-Gly-Arg-pNAxHCl = S-2444 (Trypsin) wurden von der Deutschen Kabi GmbH, Levelingstr. 18, D-8000 München 80 gekauft. Tos-Gly-Pro-Lys-pNA·CH₃-COOH (Chromozym PL) (Plasmin) stammte von Boehringer, Mannheim.

40

HPLC und FPLC

Präparative und analytische HPLC- sowie FPLC-Chromatografien wurden mit geeigneten Säulen durchgeführt.

45

Aminosäuresequenzbestimmungen

Für die Bestimmung der Aminosäuresequenzen der erfindungsgemäßen Proteinaseinhibitoren wurden 0,5 bis 2 nMol Substanz auf ein mit Polybren® vorbehandeltes Glasfaserfilter geladen und die Sequenzanalyse mit dem Gasphasen Protein Sequenzer (Fa. Applied Biosystems 470) durchgeführt. Die in jedem Zyklus freigesetzten Aminosäure-phenylthiohydantoine wurden mittels HPLC an einer Zorbax CN-Säule durch isokratische Elution nach Beyreuther (K. Beyreuther, B. Biesler, J. Bovens, R. Dildrop, K. Neifer, K. Stüber, S. Zais, R. Ehrling und P. Zabel in: Modern Methods in Protein Chemistry S. 303-325; 1983, Walter de Gruyter, Berlin) identifiziert und quantifiziert.

55

Bestimmung der quantitativen Aminosäurezusammensetzung

- Jeweils ca. 1 nMol Inhibitor wurde nach J.T. Potts jr. - Anal. Biochem. 131, 1-15 (1969) - mit 200 µl konstant siedender Salzsäure, die 0,05 % Mercaptoethanol enthielt, in einem abgeschmolzenen evakuierten Pyrexröhrchen 22 Stunden auf 110 °C erhitzt. Die Eindampfdruckstände wurden in 150 µl 0,2 M Natriumcitratpuffer pH 2,2 aufgenommen und unlösliche Anteile durch Filtration abgetrennt. Die in den Hydrolysaten vorliegenden Aminosäuren wurden in einem Biotronic LC 5000 Aminosäureanalysator getrennt, der mit einem Fluoreszenzdetektor und einem Shimadzu CR 2AC-Integrator ausgerüstet war. Die Quantifizierung erfolgte nach Derivatisierung mit o-Phthaldialdehyd, wie von J.R. Benson und P.E. Hare - Proc. Nat. Acad. Sci. 72, 619-622 (1975) - beschrieben.

Polyacrylamidalelektrophoresen

- Die SDS-Gelelektrophoresen wurden nach Laemmli -U.K. Laemmli, Nature 277, 680 (1970) - durchgeführt. Die Proteinbanden wurden angefärbt, wie von J.L. Stephano, M. Gould und L. Rojas-Galicia - Anal. Biochem. 152, 308 (1986) - beschrieben.

Renaturierung der inaktiven Inhibitoren nach der Bromcyan-Spaltung

- Die Renaturierung der nach Bromcyan-Spaltung erhaltenen inaktiven Inhibitoren erfolgte nach dem von Creighton erstmals für Aprotinin beschriebenen Verfahren (T.E. Creighton, in: UCLA Symposia on Molecular and Cellular Biology 39, 249-257; 1986, D.L. Oxender, Ed., A.R. Liss, Inc., New York).

EnzymtesteBestimmung des Inhibitorgehaltes in Fermentationsansätzen

Eine Probe der Fermentationsbrühe wurde durch Zentrifugieren geklärt und die inhibitorische Aktivität im Überstand und in den Zellen wie folgt bestimmt:

- a) Kulturfiltrat: 1 ml Kulturfiltrat wurde mit 10 µl einer 5 %igen wäßrigen Lösung von Tween 80 und 40 µl Perchlorsäure (70 %ig) unter gutem Durchmischen versetzt. Man hielt die Mischung 30 Minuten bei Raumtemperatur und trennte den gebildeten Niederschlag durch Zentrifugieren ab. Die überstehende klare Lösung wurde durch Zugabe von 180 µl gesättigter Trislösung neutralisiert. Zu 420 µl 0,2 M Tris-Salzsäure-Puffer pH 8,0, der 0,5 % Natriumazid und 0,1 % Tween 80 enthält, fügte man 8 µl einer frisch hergestellten Lösung von humaner Granulozytenelastase - erhalten durch einhundertfache Verdünnung einer Stocklösung von 1 mg Enzym je ml 0,05 M Natriumacetatpuffer pH 5,5 (Ethylenglykol 1:1 mit Testpuffer) und in einer Konzentrationsreihe die gewünschten Volumina des neutralisierten Fällungsüberstandes. Dann wurde in jeder Probe das Volumen mit Testpuffer auf 550 µl ergänzt, wobei die 100 % - Probe nur Testpuffer enthielt. Nach 30 Minuten Vorinkubation bei Raumtemperatur wurde mit jeweils 100 µl einer Mischung von Testpuffer und 6,5 µl einer 0,1 M Lösung von MeOSuc-Ala-Ala-Pro-Val-pNA versetzt und der durch die enzymatische Freisetzung von p-Nitroanilin bedingte Extinktionsanstieg bei 405 nm bestimmt. Die prozentuale Inhibition errechnet sich nach:

$$\% \text{ Inhibition} = 100 \times \left[1 - \frac{\text{OD mit Inhibitorzusatz}}{\text{OD ohne Inhibitorzusatz}} \right]$$

Die quantitative Ermittlung der Inhibitorgehalte erfolgte unter Verwendung der mit rekombinanten Val-15-Aprotinin erstellten Eichkurven.

- b) Zellen: Die beim Zentrifugieren der Kulturbrühen erhaltenen Zellen wurden in 1/10-Originalvolumen 50 mM Tris-HCl-Puffer pH 7,5 aufgeschlämmt, der 0,05 %ig an Tween 80 war. Die Zellen wurden unter Verwendung eines Ultraschallgerätes (Branson Sonifier, Cell Disruptor B 15) bei 4 °C aufgeschlossen (400 Watt, 1 Minute Beschallung je ml Probenvolumen). Nach der Fällung der

endogenen Inhibitoren mit Perchlorsäure in der oben beschriebenen Weise wurde der Inhibitorgehalt analog bestimmt.

5 Ermittlung der Hemmspezifität der Inhibitoren

Die Bedingungen für die Testung der Reinsubstanzen auf ihre inhibitorische Aktivität für die Enzyme Cathepsin G, Granulozytenelastase, Organ-(Harn)kallikrein, Plasmakallikrein und der Trypsine sind in Tabelle 1 zusammengestellt. Die Bestimmung der Enzymaktivitäten sowie die Hemmung der Enzyme wurden
10 nach an sich bekannten Verfahren durchgeführt.

15

20

25

30

35

40

45

50

55

Tabelle 1

| Bedingungen für die Testung diverser humaner Serinproteininasen | | | | | |
|---|----------------------|--|---|----------------------|---------------------|
| Enzym | [Eo] (M) | Testpuffer* | Substrat | [So] (M) | Vorinkubation (min) |
| Cathepsin G (human) | 2×10^{-7} | 0,2 M Tris; 50 mM MgCl ₂ ; 0,05 % Tween 80, pH 7,2 | MeOSuc-Ala-Ala-Pro-Met-pNA ¹⁾ | 1×10^{-3} | 60 |
| Granulozytenelastase (human) | 5×10^{-9} | 0,2 M Tris; 0,5 % Tween 80, pH 8,0 | MeOSuc-Ala-Ala-Pro-Val-pNA ¹⁾ | 3×10^{-4} | 180 |
| Organkallikrein (human) (Harn) | $2,5 \times 10^{-8}$ | 0,2 M Tris; 0,05 % Tween 80, pH 8,2 | D-Val-Leu-Arg-pNAX2HC ²⁾ | $1,5 \times 10^{-4}$ | 30 |
| Plasmakallikrein (human) | 3×10^{-9} | 0,2 M Tris; 0,05 % Tween 80, pH 8,2 | D-Pro-Phe-Arg-pNAX2HC ³⁾ | 4×10^{-4} | 120 |
| Plasmin (human) | 5×10^{-9} | 0,2 M Tris/0,01 M CaCl ₂ ; 0,05 % Tween 80, pH 8,0 | Tos-Gly-Pro-Lys-pNA-CH ₃ -COOH ⁴⁾ | 1×10^{-4} | 90 |
| Trypsin (Rind) | 1×10^{-9} | 0,2 M Tris; 0,01 M CaCl ₂ ; 0,05 % Tween 80, pH 8,0 | Pyr-Gly-Arg-pNA ⁴⁾ | 2×10^{-5} | 90 |
| Trypsin anionisch (human) | 1×10^{-9} | 0,2 M Tris; 0,01 M CaCl ₂ ; 0,05 % Tween 80, pH 8,0 | Pyr-Gly-Arg-pNA ⁴⁾ | 2×10^{-5} | 90 |
| Trypsin cationisch (human) | 1×10^{-9} | 0,2 M Tris; 0,01 M CaCl ₂ ; 0,05 % Tween 80, pH 8,0 | Pyr-Gly-Arg-pNA ⁴⁾ | 2×10^{-5} | 90 |

* alle Testpuffer enthielten 0,05 % NaN₃¹⁾ K. Nakajima, J.L. Powers, B.M. Ashe und M. Zimmermann, J. Biol. Chem. 254, 4027-4032 (1979)²⁾ E. Ammundsen, J. Pütter, P. Friberger, M. Knos, M. Larsson und M. Claesson, Adv. Exp. Med. Biol. 120A (1979)³⁾ A.M. Vennéröd, K. Laake, A.K. Solberg und S. Strömblad, Throm. Res. 9, 457 (1976)⁴⁾ B. Wiman, Throm. Res. 17, 143 (1980)⁵⁾ G. Claesson et al., Haemostasis 7, 76 (1978)

Konstruktion von Vektoren für die Expression von API-Homologen in Hefe

5 Das Ausgangsplasmid für die Konstruktion von pMT15 ist pCGS65 von C.A. Kaiser, Massachusetts Institute of Technology, Cambridge, MA. Das Plasmid trägt als Selektionsmarker Amp^R und URA3 sowie den Replikationsorigin von pBR322 und ein Segment von 2 μ , wodurch dieses Plasmid in S.cerevisiae und E.coli replizieren kann. pCGS65 enthält die gesamte cDNA von SUC2 (Invertase). Durch BamHI-Spaltung
 10 wird das COOH-terminale Ende der Invertase deletiert und durch ein 160bp BamHI-BglII-Fragment von URA3 ersetzt, welches als Transkriptionsterminator in Hefe wirkt (Yarger et al. Mol.Cell.Biol. 6, 1095-1101; 1986). Das so entstandene Plasmid pIT18 wurde mit EcoRI und BamHI gespalten, wodurch ein 1700bp-Fragment des SUC2-Promoters und die NH₂-terminale Hälfte der Invertase deletiert wurde. Dieser Teil wurde ersetzt durch ein 1200bp EcoRI-BamHI-Fragment aus pJS12 (R.C. Das et al. Mol.Gen.Genet. 1989 in
 15 press), welches den MF α 1 Promoter und die pre-pro-Leadersequenz des α -Faktors enthält zusammen mit den 34 NH₂-terminalen Aminosäuren der Invertase. Hieraus entsteht pMT15 als Ausgangsvektor für die Expression von API-Homologen in Hefe. Die Gene der API-Homologen wurden als 180bp HindIII-Fragment (siehe Version 1, Abb. 2) in die HindIII-Schnittstelle von pMT15 kloniert.

20

Hefe-Transformation

100 ml Hefezellen von SC106 (S2207A von Yeast Genetics Stock Center, University of California, Berkeley, CA 94720) werden in SD-Medium (0,67 % yeast nitrogen base without aminoacids und 2 %
 25 Glucose) supplementiert mit jeweils 20 mg/l Threonin, Methionin und Histidin bei 30° bis 2 x 10⁷ Zellen/ml gezüchtet. Die Zellen werden durch Zentrifugation geerntet, 1 x mit 5 ml TE (10 mM Tris-HCl pH 7,5, 1mM EDTA) und 1 x mit 5 ml TE + 0,1 M Lithiumacetat gewaschen. Das Zellpellet wird in 1 ml 0,1 M Lithiumacetat suspendiert und für 1 Stunde bei 30° C inkubiert. Die kompetenten Zellen können bis zu 2 Tagen bei 4° C aufbewahrt werden. 0,1 ml Zellen werden mit 10 μ l Plasmid-DNA (ca. 5 μ g) und 15 μ l
 30 denaturierter Heringsperm-DNA (3 mg/ml) versetzt. Nach 30 Minuten bei 30° C werden 0,7 ml TE (40 % PEG 3350, 0,1 M LiOA) hinzugegeben und 1 Stunde bei 30° C inkubiert. Die Zellen werden für 5 Minuten auf 42° C erhitzt. Anschließend wird das Zellpellet 2 x mit 0,5 ml TE gewaschen. Das Zellpellet wird in 0,1 ml TE suspendiert und auf Agarplatten mit Selektionsmedium ausgestrichen. Transformanten erscheinen nach 3 Tagen.

35

Kulturbedingungen

Transformierte Hefezellen werden in SD-Medium (supplementiert mit jeweils 20 mg/l Threonin, Histidin
 40 und Methionin) bei 28° C oder 30° C über einen Zeitraum von bis zu 96 Stunden fermentiert. Die Zellen werden durch Zentrifugation geerntet und die inhibitorische Aktivität der API-Homologen wird im Medium gemessen.

45 **Ansprüche**

1) Peptid, enthaltend im wesentlichen die Aminosäuresequenz des Alzheimer Protease Inhibitors (API), wobei mindestens eine Aminosäure der natürlichen Sequenz durch eine andere natürliche Aminosäure ersetzt worden ist.

50 2) Peptide gemäß Anspruch 1, mit einem Aminosäureaustausch in Position 13 und/oder 15, 37, 50.

3) Peptide gemäß den Ansprüchen 1 und 2 mit einem oder mehreren der folgenden Austausche

Position 13

Val, Ile, Leu, Ala, Met, Asn, Gln, Phe, Tyr oder Trp

Position 15:

55 Leu, Gly, Ala, Phe, Arg, Val, Ile, Ser, Thr, Asp, Glu, Asn, Gln oder Tyr

Position 37:

Arg, Val, Ile, Leu, Met, Asn, Gln, Asp, Glu, Phe, Ser oder Thr

Position 50:

Val, Ile, Leu, Gln, Glu, Thr, Asp, Phe, Lys oder Arg.

4) Arzneimittel, enthaltend ein oder mehrere Peptide der Ansprüche 1 bis 3.

5) Verwendung der Peptide aus den Ansprüchen 1 bis 3 als Protease Inhibitoren.

6) Desoxyribonucleinsäure codierend für eines der Peptide gemäß der Ansprüche 1 bis 3.

5 7) Expressionsvektor enthaltend die Desoxyribonucleinsäure gemäß Anspruch 6.

10

15

20

25

30

35

40

45

50

55

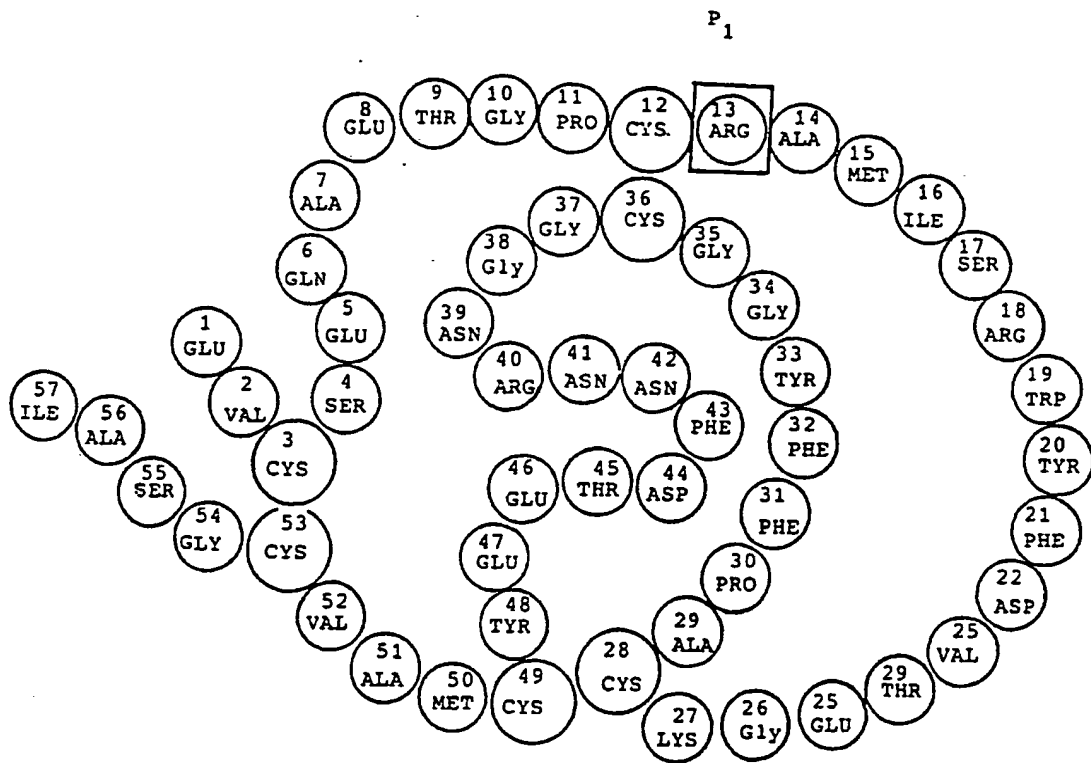


FIG.1

Hind III
 ↓
 A GCT GAG GTC TGC AGT.....
 CTC CAG ACG TCA.....
 Version 1: - Ala- Glu- Val - Cys - Ser -
 Pst I
 ↓
 Nhe I
 ↓
 Version 2: CT AGC GAG GTC TGC AGT GAA CAA GCC GAG ACG GGC CCG TGC CGA GCA ATG ATA TCC
 G CTC CAG ACG TCA CTT GTT CGG CTC TGC CCG GGC ACG GCT CGT TAC TAT AUG
 Pst I
 ↓
 Eco RV
 ↓
 Glu-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-Pro-Cys-Arg-Ala-Met-Ile-Ser-
 Acy I
 ↓
 Bsh I
 ↓
 CGC TGG TAC TTT GAC GTC ACT GAA GGG AAG TGC GCG CCA TTC TTT TAC GGC GGA TGT
 GCG ACC ATG AAA CTG CAG TGA CTT CCC TTC ACG CGC GGT AAG AAA ATG CCG CCT ACA
 -Arg-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-
 Xmn I
 ↓
 Sal I
 ↓
 Bam III
 ↓
 Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly
 Hind III
 ↓
 TCC GCC ATT TGA TA
 AGG CGG TAA ACT ATT CGA
 Ser Ala Ile xxx xxx

FIG.2



Europäisches
Patentamt

EUROPÄISCHER RECHERCHENBERICHT

Nummer der Anmeldung

EP 90 10 6529

| EINSCHLÄGIGE DOKUMENTE | | | |
|--|---|--|---|
| Kategorie | Kennzeichnung des Dokuments mit Angabe, soweit erforderlich, der maßgeblichen Teile | Betrifft Anspruch | KLASSIFIKATION DER ANMELDUNG (Int. Cl.5) |
| A | EP-A-0 304 013 (ASAHI KASEI KOGYO K.K.) --- | | C 07 K 7/10 A 61 K 37/64 C 12 N 15/15 |
| A | EP-A-0 297 362 (BAYER AG) ----- | | |
| | | | RECHERCHIERTE SACHGEBIETE (Int. Cl.5) |
| | | | C 07 K A 61 K C 12 N |
| Der vorliegende Recherchenbericht wurde für alle Patentansprüche erstellt | | | |
| Recherchenort DEN HAAG | | Abschlußdatum der Recherche 26-07-1990 | Prüfer DEFFNER C-A.E. |
| KATEGORIE DER GENANNTEN DOKUMENTE | | | |
| X : von besonderer Bedeutung allein betrachtet Y : von besonderer Bedeutung in Verbindung mit einer anderen Veröffentlichung derselben Kategorie A : technologischer Hintergrund O : mündliche Offenbarung P : Zwischenliteratur | | T : der Erfindung zugrunde liegende Theorien oder Grundsätze E : älteres Patentedokument, das jedoch erst am oder nach dem Anmeldedatum veröffentlicht worden ist D : in der Anmeldung angeführtes Dokument L : aus anderen Gründen angeführtes Dokument & : Mitglied der gleichen Patentfamilie, übereinstimmendes Dokument | |